PHYSICAL TECHNIQUES
IN
BIOLOGICAL RESEARCH

Volume I

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Physical techniq.

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optical techniques,
photochemistry, luminescence,
light scattering, light,
Absorption spectroscopy,
Spectrophotometry,
microscopes, microscopy,
Birefringence, Dichroism,

PHYSICAL TECHNIQUES IN BIOLOGICAL RESEARCH

Volume I

Optical Techniques

PHYSICAL TECHNIQUES IN BIOLOGICAL RESEARCH

Volume I. OPTICAL TECHNIQUES
Volume II. PHYSICAL CHEMICAL TECHNIQUES
Volume III. CELLS AND TISSUES

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PHYSICAL TECHNIQUES
IN
BIOLOGICAL RESEARCH

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Volume I

OPTICAL TECHNIQUES

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PREFACE

These volumes have resulted from collaborative effort, in which a large number of physical techniques that have been found useful in biology are discussed briefly from both the theoretical and the practical viewpoint. No claim to encyclopedic range is made, but it is believed that from these volumes the reader can get a fairly comprehensive idea of the present place of the techniques of physics and of physical chemistry in biological research.

During the last two decades there has been unprecedented broadening of the scope of attack on fundamental problems of physiology. This is largely a consequence of the increasing use by biologists of modern physical techniques, some of which are of the most advanced types and characterized by precision and delicacy rarely employed in their application in physics. These refinements have not always resulted from the collaboration of a biologist and a physicist, as one might suppose. Fully as often the biologist has turned physicist and has himself adapted, or indeed sometimes developed, physical techniques suited to his needs. Thus, Martin and Synge developed adsorption experimental procedures into the enormously useful paper chromatography; Holter and Linderstrøm-Lang made from the diver of Descartes an apparatus delicate enough to obtain analytical data from single cells; while in the microtome electron microscopists have designed an engineering marvel that cuts slices about one order of magnitude above the range of monomolecular films.

Workers with any of the powerful new aids to biology from physics cannot avoid feeling strongly encouraged to continue to elaborate new methods and to broaden the range of biological problems to which the techniques are applicable. The present work has been conceived in the hope of accelerating such development and wider use. Every specialist in one of these techniques is constantly called upon to help his fellow biologist to decide whether, or how, a particular physical technique can serve a biological use more or less unlike that for which it was originally designed. It is also not an infrequent experience of a physicist or a physical chemist to have a biologist come to him with a difficulty which he hopes can be overcome if he can find the right physical approach. The success of such conferences depends to a large extent upon both participants having a fair knowledge of the details of the techniques and the biological uses to which they have already been put; and one of the aims of the present work is to serve such a need. For every such biologist who makes a vigorous effort to use new physical methods, there must be many who have a vague feeling that their researches might proceed better if reoriented in a physical direction, but who hesitate to attempt this because of timidity. For such workers it is felt that the many examples of simple methods will be helpful. Finally, in a broad sense, it is hoped that this treatise will serve as a real orientation for biologists and for chemists and physicists who may be potential biologists. In these volumes each author, an expert in his field, has written in such a way that a biologist can see whether he may start to employ the technique, or whether the application to his particular biological problem demands collaboration with a physicist or a physical chemist. The latter, on the other hand, should be able to assess in realistic terms the possibility of fruitful and exciting application of his special training to the baffling problems of biology.

The arrangement of material has been determined primarily by biological considerations. Volumes I and II deal with theory and methods applied to relatively pure preparations of biological substances that are obtained from cells or other tissue elements. The optical approaches, so favored in biology, are in Volume I, while in Volume II are a wide variety of nonoptical techniques (the only exception is the chapter on X-ray diffraction which is in Volume II). Volume III deals with the application of physical techniques to cells and tissues.

The editors wish to express their appreciation to the authors for their contributions.

New York, N. Y.
June 30, 1955

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Arthur W. Pollister

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I. Principles of Photochemistry

1. THE LIGHT ABSORPTION ACT

Photochemistry is the study of chemical reactions initiated by radiant energy. The statement that the energy must be absorbed in order to induce chemical events is often called the first law, or Grotthus' law, of photochemistry. Radiant energy is absorbed by matter in discrete amounts called quanta. The energy of a single quantum, ϵ, is proportional to the frequency of the radiation, ν.

$$\epsilon = h\nu \tag{1}$$

Planck's constant, h, has the numerical value of 6.62×10^{-27} erg seconds. A more practical unit for the biologist is the energy of a mole of quanta, or an einstein, $N\epsilon$, where N is Avogadro's number, 6.02×10^{23}. In more familiar terms, the energy of an einstein in kilocalories per mole, E, is related to the wave length in Angstrom units, λ, by equation (2).

$$E = \frac{2.859 \times 10^5}{\lambda} \tag{2}$$

a. Molecular Energy Levels

Absorption of light by a molecule results in a conversion of radiant energy into the energy of internal motions within the molecule. These motions can be classified as rotations of the molecule as a whole, vibrations of the atomic nuclei with respect to each other (bond stretching or bending), and motions of the electrons. The ground state of a molecule is that state in which the energies of all these motions have their minimum values. Other states of the molecule are called excited states, and the transition of a ground state molecule to an excited state with the absorption of energy is called excitation. The reason that only certain wave lengths are absorbed by a given type of molecule is that the energies of these internal motions can have only certain discrete values, and the energy of an absorbed quantum must exactly equal the excitation energy for some allowed excited state. The excitation energy is equal to the difference in the total internal energy of the molecule between the excited and ground states.

Ordinary chemical reactions take place in the dark when a molecule absorbs a critical amount of vibrational energy as the result of collisions with other molecules. If the energy must be absorbed in the form of light, as is the case in photochemical reactions, it is necessary that excitation occur in the electronic motions. The spectral regions in which molecular electronic absorption occurs are the ultraviolet, the visible, and the very near infrared. For polyatomic molecules the transition to an excited electronic configuration is characterized by a fairly broad absorption band, in which varying degrees of vibrational and rotational excitation are superimposed upon a fundamental electronic excitation.

b. Action Spectra

If we are confined to the easily accessible near ultraviolet, above 2400 A, then the molecular groupings giving rise to the important absorptions are the familiar chromophores: unsaturated linkages, aromatic compounds, and other resonating structures. Thus, proteins are most likely to be photochemically active at the tyrosine, tryptophane, and phenylalanine residues; nucleic acids are universally ultraviolet sensitive because of the purine and pyrimidine moieties; and so on. The absorption spectrum thus provides a suggestive pattern in choosing the wave length for a photochemical reaction. In many biological processes, however, the situation is reversed. That is, a photochemical phenomenon is observed before the definite identity of the active absorbing molecule is known. In such a case it is very useful to measure the wave length dependence of the phenomenon. The plot of the quantitative biological or chemical response as a function of wave length is called the action spectrum. In order to make measurements for such a plot a dilute sample should be chosen which absorbs only a small fraction of the light incident upon it. The experiment must be performed at various more or less narrow wave length regions. The two common conventions for recording such data are either (1) the amount of response per unit amount of incident energy, or (2) the amount of response per unit number of incident light quanta. The energy multiplied by the average wave length in the selected light region gives a product which is proportional to the number of quanta. If the total wave length span of the action spectrum is not large, the two curves will have almost the same shape. The wave length correction inherent in the type (2) curve makes this a convenient form for comparison with the absorption spectrum, plotted as the extinction coefficient against wave length. For purposes of comparison the values of the ordinates on the action spectrum should all be multiplied by a constant normalizing factor chosen so that the action spectrum will have the same numerical value as the absorption spectrum at some arbitrarily chosen wave length. Then if the photochemical response, or action, is proportional to the number of quanta absorbed, irrespective of wave length, the normalized type (2) action spectrum determined at less than 10% absorption will coincide with the absorption spectrum at all wave lengths. This can be shown in Eqs. (3) and (4), where D is the optical density, k is a constant, I_0 is the incident intensity, I is the transmitted intensity, and I_{abs} is the absorbed intensity.

$$\text{action per unit of incident radiation} = \frac{kI_{\text{abs}}}{I_0} = k\left(1 - \frac{I}{I_0}\right) \tag{3}$$
$$= k(1 - 10^{-D}) = k(1 - e^{-2.3D})$$

When D is very small this expression can be simplified by MacLaurin's theorem.

$$\text{action per unit of incident radiation} = 2.3kD \tag{4}$$

Since D is proportional to the extinction coefficient, the action per unit of incident radiation is also proportional to the extinction coefficient.

The useful feature of the action spectrum is that it has the shape not of the absorption spectrum of an overall mixture but of the absorption spectrum of only those components that absorb energy used in the photochemical reaction. If there is only one active component it can often be identified directly in this manner. Thus the action spectrum for human scotopic vision can be fitted to the absorption spectrum of rhodopsin (Hecht, 1944); the action spectrum for phototaxis in *Rhodospirillum rubrum* can be fitted to the absorption spectrum of bacteriochlorophyll (Milatz and Manten, 1953); and the action spectrum for the photoreactivation of *Streptomyces griseus* previously exposed to ultraviolet light can be fitted to the absorption spectrum of a porphyrin (Kelner, 1951). There are often more complicated cases where more than one component may contribute absorbed energy for the photochemical purpose or where an active and an inactive component may both absorb at the same wave length. An example is an analysis of the relative roles of various plastid pigments in photosynthesis (Emerson and Lewis, 1943).

2. Utilization of Absorbed Light Energy

There are several different courses that the energy of absorption may take after it has appeared as internal energy of molecular excitation. That the energy does not remain long in the absorbing molecule is proved by the simple experimental fact that the color of most substances does not change during a period of illumination. This must mean that the excited molecules are quickly returned to the ground state where they are prepared to absorb the same wave lengths as at the first instant of irradiation.

a. *Fluorescence*

The simplest type of dissipation of excitation energy is the emission of light. Such an emission, if it occurs very soon after the absorption, less than about 10^{-6} seconds, is called fluorescence. The ratio of the number of quanta emitted as fluorescence to the number of quanta absorbed is known as the fluorescence yield. Even if the fluorescence yield is one, there are probably no cases of organic molecules in solution or in tissues which can convert 100% of the absorbed energy into fluorescent light. This is because the average fluorescent quantum is at a longer wave length and thus has less energy than the absorbed quantum. The peak of the emission spectrum for fluorescence usually occurs at longer wave lengths than the peak of the absorption spectrum, and the tail of the fluorescence curve spreads out even further toward the red. This red shift, and hence energy deficiency, of the fluorescent light is explained in terms of the Franck-Condon principle. According to this principle the most probable electronic transitions are those for which the average interatomic distances are the same in the initial and final states. The absorption of light does not necessarily bring a molecule to the lowest vibrational state in the new electronic configuration because the interatomic distances in that state are not necessarily the same as in the ground state. Instead, the primary excitation will leave the molecule in a vibrating state for which the average interatomic distances correspond most closely to the distances in the ground state of the molecule. The excess vibrational energy in the higher electronic state will be dissipated as heat by rapid impacts with the solvent or other surrounding molecules. Fluorescence will then occur mainly from the lowest vibrational level of the excited state and the fluorescence transition will occur preferentially to a vibrating state having the proper match of interatomic distances. The excess vibrational energy of the molecule will again be dissipated to the milieu as heat. Another important aspect of the fluorescent spectral band is that it is independent of the absorption frequency selected for excitation. If a molecule has an absorption band in the near ultraviolet and another in the green, the fluorescence will be only in the green, slightly displaced by the usual shift. The radiationless transition from the second to the first electronic excited state occurs by internal conversion, discussed in *c*, below.

b. Phosphorescence

Many organic molecules, particularly those with highly conjugated structures, have in addition the ability to radiate after passing through an electronic state intermediate in energy between the normal ground state and the fluorescent state. This type of luminescence, called phosphorescence, occurs with a time delay of as much as several seconds after the absorption act. The phosphorescent light at normal temperatures has the same spectrum as fluorescence, but at low temperatures a new luminescence spectrum may appear at a considerably longer wave length. The first type of phosphorescence results from the non-radiative transition from the phosphorescent state to the fluorescent state. For several cases the phosphorescent state has been shown to be a triplet state, that is, a biradical in which two electrons have unpaired spins. The importance of the phosphorescent state in biology is that it is much more reactive chemically than the fluorescent state, both on account of its free radical nature and

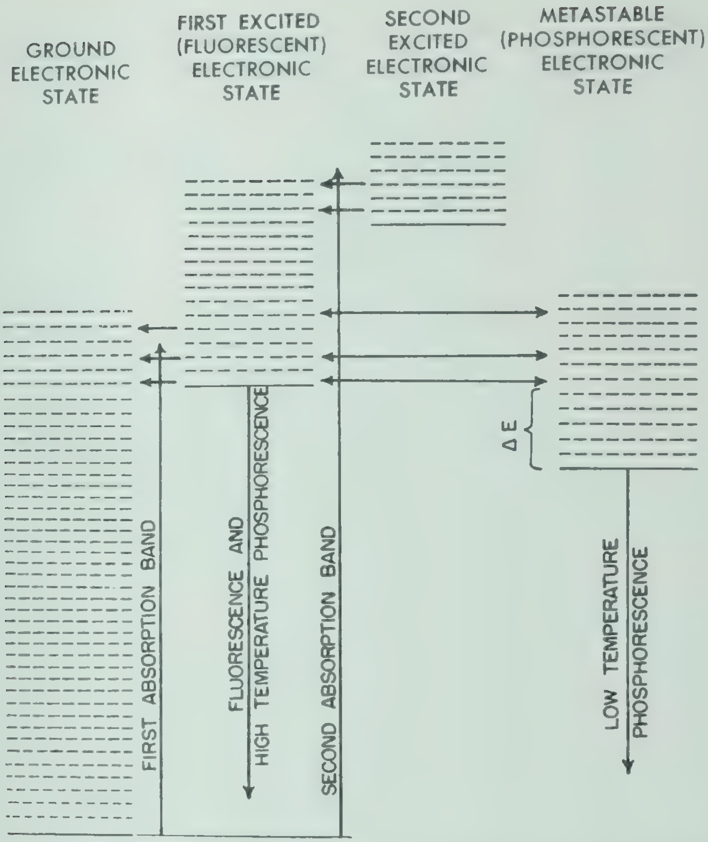
GROUND ELECTRONIC STATE
FIRST EXCITED (FLUORESCENT) ELECTRONIC STATE
SECOND EXCITED ELECTRONIC STATE
METASTABLE (PHOSPHORESCENT) ELECTRONIC STATE
FIRST ABSORPTION BAND
FLUORESCENCE AND HIGH TEMPERATURE PHOSPHORESCENCE
SECOND ABSORPTION BAND
ΔE
LOW TEMPERATURE PHOSPHORESCENCE

FIG. 1. Schematic diagram of molecular energy levels. This is an energy level diagram in which vertical distances are proportional to the differences in internal energy between various states. Solid horizontal lines represent the electronic states without excess vibrational excitation. The broken lines represent varying degrees of vibrational excitation superimposed on the electronic energies. Each upward arrow represents the strongest transition within an absorption band. Each downward arrow represents the strongest transition within an emission band. The length of each vertical arrow on the energy scale is related by Eq. (2) to the wave length at the peak of the absorption or emission band. The horizontal arrows represent internal conversion transitions. ΔE represents the minimum energy dissipation in the passage from the fluorescent to the phosphorescent state.

also because of its relatively long life. For example, most of the dyes that sensitize the photochemical oxidations constituting photodynamic action have well-known phosphorescent properties (Blum, 1941). The relationship between absorption, fluorescence, and phosphorescence is illustrated graphically in Fig. 1.

c. *Internal Conversion*

Vibrational dissipation of absorbed energy is observed not only as part of the emission process but often in exclusion to any luminescence. Most organic molecules in solution have a fluorescence yield considerably less than one. This means in most cases that much of the energy of electronic excitation is dissipated as heat. This occurs because of the internal conversion from a higher electronic state to a lower electronic state (Franck and Livingston, 1941). This conversion occurs between combined electronic-vibrational states of equal energy and is followed by stepwise dissipative losses of the excess vibrational energy to the medium. The chemical consequences of internal conversion are very important because the high content of vibrational energy following this process produces a "hot molecule," the type produced by high temperature collisional activations. Thus a photochemical reaction could result in products similar to ordinary reactions, but now the light replaces thermal agitation as the source of the energy of activation.

It might appear at first glance that most molecules should decompose if a quantum of absorbed visible or ultraviolet light should be completely converted internally, since such quanta have energies, calculated by Eq. (2), in the range of the typical chemical bond, 50 to 150 kcal. Actually, simple bond rupture is a rare consequence of internal conversion because rupture requires the localization of the requisite energy in a particular bond. The vibrational energy of a molecule after internal conversion is distributed over many possible modes of vibration of a variety of atoms in the molecule. Especially for complicated molecules the amount of vibrational energy in any one pair of adjacent atoms is a small fraction of the total energy. Rather than simple bond rupture, another type of chemical result of internal conversion requiring less energy is an intramolecular rearrangement in which bonds are both broken and formed or in which stereo-isomerization occurs by rotation about a rigid bond (Franck and Sponer, 1948). The overall energy requirement for such a process is not so large as for a simple dissociation.

It has been known for a long time that *cis-trans* isomerization often occurs in ultraviolet light. At a given temperature and wave length a photostationary state can be established in which there is a constant ratio of *cis* to *trans*. In general this stationary distribution will not be the same as the equilibrium distribution achieved in the dark with a catalyst. Some important biological reactions involve such a photo-isomerization. One of these, the isomerization of vitamin A aldehyde, retinene, will be discussed in Section 3*b*. (Hubbard and Wald, 1952). Kuhn and Winterstein (1933) reported the complete conversion in the light of the carotenoid, *cis*-crocetin dimethyl ester to the *trans*-isomer. This reaction is reported to be one of the principal mechanisms by which light exercises an influence on the mating characteristics of the green alga, *Chlamydomonas* (Kuhn *et al.*, 1938; Moewus, 1950).

Another chemical consequence of photochemically produced hot molecules has been proposed by Franck and Platzman (1954). They believe that the heating in the immediate neighborhood of a hot molecule, resulting from the absorption of radiation, might cause local disturbances of the intermolecular structure in biological tissues. According to their picture, the hydrogen bonds which are responsible for the shape of protein molecules might easily be vulnerable to such heating effects. It is important to point out here that the energy of excitation, initially located in the electronic system of a particular absorbing chromophoric group, may spread quickly after internal conversion, by the strong coupling of molecular vibrations, to the extremities of the molecule and even to the first sphere of the solvated molecules of the environment.

d. Chemical Reaction

Photochemists usually use a separate term for those reactions in which the electronic energy of absorption is transferred, without general dissipation, into the vibrational energy of a particular bond. A resulting bond rupture is called dissociation or pre-dissociation. If the molecule were a normal saturated structure, the primary products would be free radicals.

Methods exist for detecting the intermediacy of free radicals in photochemical reactions. Free radicals are known to initiate polymerization processes in solution. If a reaction mixture containing a monomeric substance is resistant to polymerization in the dark but undergoes polymerization in the light, the photoproduction of free radicals is strongly suggested. Uri (1952) has used this technique to demonstrate radicals in some photochemical reactions of chlorophyll. In applying this method it is necessary to choose a wave length that is not absorbed by the test monomer. Sometimes, chemical reactions of free radicals can be used for identification. Bawn and Mellish (1951) have used the stable radical, diphenylpicrylhydrazyl, as a trapping reagent for certain other radicals. The reagent is highly colored and is bleached upon reaction with another radical. The presence of benzoyl peroxyl radicals can be shown in this manner. Calvin and Barltrop (1952) have used the same reagent to test for a biradical formed by the rupture of a disulfide bond when zinc tetraphenylporphin was illuminated in the presence of *n*-propyldisulfide. Calvin (1953) has used the dehydrogenation of tetralin as another test for the photosensitized formation of the disulfide biradical. In any of these tests for photochemically produced radicals that require the addition of a test reagent to the reacting system, controls must always be made to be sure that the reagent does not interfere with the primary reaction being investigated. Sometimes, bond rupture does not lead to free radicals, but to stable molecules. An example is the photochemical liberation of carbon monoxide from carbon monoxide-iron porphyrin complexes (Warburg and Negelein, 1928). In all cases of bond rupture, it must be emphasized that the particular bond that is ruptured is not necessarily the closest bond to the absorbing chromophoric group. Because of the coupling of the molecular vibrations and the complexity of the excited electronic state of polyatomic molecules, it is difficult to predict in advance which, if any, bond will break. It should be no surprise, therefore, that absorption by a phenylalanine moiety in a polypeptide results in the rupture of a peptide bond several atoms removed from the phenyl ring (McLaren, 1949). It is quite unnecessary to invent an explanation of this reaction in terms of the very unlikely migration of an electron through the molecule.

The first law of thermodynamics provides a necessary, but of course not sufficient, condition for bond rupture. In other words the wave length of the light must be low enough to satisfy the requirement of the bond dissociation energy according to Eq. (2). Table I lists some bond dissociation energies, based on measurements with simple molecules. The values for this table are based on a review by Szwarc (1950) and on measurements of Franklin and Lumpkin (1952). Such a table should be used only for approximation because the energy for a given type bond is not constant from molecule to molecule.

TABLE I

Bond Dissociation Energies (in kcal. per mole)

O—H	118	C—N (amide)	98
S—H	87	C—N (amine)	77
N—H	104	S—S	64
C—H (aliphatic, primary)	100	C—S	72
		C—O	90
C—C	82	C═O	145

Bimolecular reactions in which the molecule after absorbing radiation transfers its excitation energy to a second molecule are termed collisions of the second kind. It is sometimes difficult to make a sharp distinction between uni- and bimolecular reactions when any molecule, as in a condensed system, is in a permanent state of impact with its surroundings. If the overall reaction involves permanent changes in both the absorber and the second molecule, the two possibilities are (1) unimolecular bond rupture followed by a reaction in the dark between one of the fragments and the second molecule; and (2) chemical rearrangement within a bimolecular complex formed by collision of the excited molecule with the second molecule. Hydrogen transfer is a particularly common type of photochemical reaction involving two molecules. Chlorophyll is known to be formed in higher plants by the photochemical dehydrogenation of protochlorophyll (Smith, 1948). Also synthetic chlorins analogous to chlorophyll in their tetrapyrrole structure undergo photochemical dehydrogenations in the presence of quinones as hydrogen acceptors (Calvin and Dorough, 1948). Shugar (1951) has postulated the photochemical transfer of hydrogen from reduced diphosphopyridine nucleotide to disulfide linkages in triosephosphate dehydrogenase to account for some observations on the irradiation of the enzyme-coenzyme complex.

e. *Electronic Energy Transfer*

(*1*) *Sensitized fluorescence.* Sensitized fluorescence is a clear-cut example of transfer of energy from the absorber to the second substance. The basic experimental observation is the emission of fluorescence by one substance during the absorption of light by another. This situation can be clearly defined experimentally if the irradiating light is absorbed by only one substance and if the luminescent light has the same spectral distribution as the fluorescence spectrum of another component. In more complicated cases where the absorption bands and/or emission bands of the different components of a mixture overlap, the interpretation of the data is more complicated (Watson and Livingston, 1950; French and Young, 1952). There are certain rules for the occurrence of sensitized fluorescence (Förster, 1948). The absorption band of the absorber must lie on the short wave length side of the absorption band of the fluorescer. Also there must be some wave length region in which the fluorescence spectrum of the absorber overlaps the absorption band of the fluorescer. Another rule regulates the maximum distance of separation of the absorber and fluorescer at which sensitization occurs. There is at the present writing some disagreement whether the two molecules must actually be in contact or may be separated by as much as 50 A. Sensitized fluorescence has been used to study the role of the auxiliary pigments in photosynthesis. The sensitization of the fluorescence of chlorophyll *a* upon the absorption of light by phycocyanin, phycoerythrin, or carotenoids is good evidence that the function of these latter pigments is to funnel energy into the photochemical apparatus by way of chlorophyll *a* (Duysens, 1952; French and Young, 1952).

(*2*) *Sensitized chemical reaction.* The sensitized chemical reaction is a photocatalytic type of bimolecular reaction. The absorber molecule transfers a large part of its excitation energy to a reacting system and is itself chemically unchanged in the overall process. This process makes it possible to extend photochemical reactions into wave length regions where the reactant itself does not absorb. Photosynthesis, phototaxis, and many types of vision are included in this category. As in the case of sensitized fluorescence, the pigment must be either closely attached to or not distantly separated from the primary chemical substrate at the instant of energy transfer.

Photosensitized oxidations with molecular oxygen constitute a special case in this category. A wide variety of substrates have been found to undergo this type of reaction with molecular oxygen (see reviews in Blum, 1941; Rabinowitch, 1945, p. 510). The light absorber is very often a fluorescent dye. The list of dyes used for this purpose includes chlorophyll, chlorophyllides, eosin, erythrosin, acriflavine, fluorescein, methylene blue, and Rose bengal. The list of substrates includes amines, hydrocarbons, thiourea, alcohols, olefins, and aromatic rings. It is very likely that the biological phenomenon known as photodynamic action represents this type of reaction (Blum, 1941). There is considerable speculation in the literature on the mechanism of these reactions, but a definitive pathway for the actual energy transfer from the dye to the reactant is not firmly established. Damage to tissues resulting from such oxidations is always a possibility when dyes are used for staining purposes.

3. Photochemical Kinetics

Certain features of photochemical kinetics are distinct from the kinetic problems of dark reactions. Only these special aspects will be considered here.

a. Intensity Dependence

(*1*) *Order with respect to light.* It is to be expected that the rate of a purely photochemical reaction should be directly proportional to the incident intensity, I_0. If p is the amount of product, then

$$dp/dt = kI_0 \tag{5}$$

On the molecular level, this means that there is a fixed probability that a molecule that has absorbed a quantum of light will lead to a reaction. The simplest test of the first order dependence on intensity is made at constant concentration of the effective absorbing substance. This condition is easily met if the reaction is a photosensitized one, in which the concentration of the absorbing pigment does not change. If the absorber undergoes chemical reaction, three possibilities exist for testing the first order law.

(a) Initial rates can be measured at different intensities. Each measurement can be performed on a fresh sample, and the measurement is made quickly enough so that the concentration of the absorber does not change appreciably.

(b) Sometimes a large excess of the absorber is present or can be supplied externally so that many measurements at different intensities may be made without causing an appreciable drop in the absorber concentration.

(c) The first order law may be restated operationally without changing the spirit of its meaning. According to the alternate statement, the rate of a reaction is proportional to the rate of absorption of radiation by the photoactive substance. If this substance is changed chemically as a result of the light reaction, then a study of the rate of decrease of its concentration, c, can be used to follow the course of the reaction.

$$-dc/dt = k'I_{\text{abs}} \tag{6}$$

For a constant incident intensity, the rate of absorption, I_{abs}, can be shown to be proportional to the concentration of active absorber as long as the total absorption is less than about 10%. Then, Eq. (6) can be rewritten as

$$-dc/dt = k''c. \tag{7}$$

In these equations, k' and k'' are constants independent of concentration. Equation (7) is the familiar differential equation from reaction kinetics for first order dependence on concentration. If this equation is obeyed, a plot of the logarithm of the concentration of unreacted material versus time should be a straight line. This type of curve is often called a survival curve because it has been used to express the inactivating or lethal effects of radiation on proteins, viruses, bacteria, and other biological systems and because biological assays of activity or survival have often been used to determine c. A straight line on the semi-logarithmic plot has often been called a one-hit curve, because it implies that the extent of reaction is proportional to the number of quanta absorbed by the "active centers." This can be explained only if one quantum is sufficient to produce the effect. There is no implication in this type of curve that each absorbed quantum will initiate the reaction, but rather that there is a fixed probability that one absorbed quantum will be effective. A given quantum thus has an either-or chance of causing reactions or being wasted by one of the dissipative processes discussed above. The so-called multi-hit survival curves look quite different and are interpreted as representing the necessary accumulation of effects of several quanta (Atwood and Norman, 1949). A single-hit curve is compared with a theoretical 10-hit curve in Fig. 2. The initial flat portion of the multi-hit curve represents the accumulation period when most of the effective quanta are preparing the way in groups of nine for the tenth and completely damaging blow that will come in the more steeply descending portion of the curve.

The experimental conditions for observing a first-order curve, as described above, are constant intensity and low fractional absorption. Another case of importance makes the method applicable for any degree of absorption. Many photosensitive biological substances continue to absorb radiation after the chemical reaction has occurred. Very often the absorption coefficient of the product is exactly the same as that for the reactant (McLaren, 1949). This has been found to be true for some enzymes and

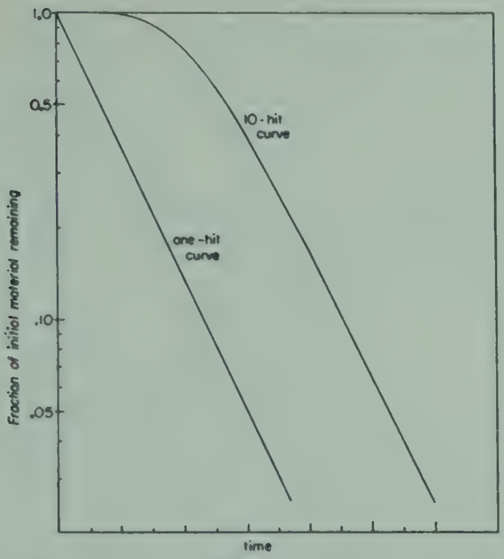

FIG. 2. Theoretical survival curves. Each curve represents the fractional number of surviving particles, S, as a function of time. The one-hit curve is calculated from the equation, $S = e^{-kt}$. The ten-hit curve is calculated from the equation, $S = 1 - (1 - e^{-kt})^{10}$. The probability, k, of a single hit per particle per unit time is the same for the two curves.

viruses. Physically, this means that the chromophoric group in the molecule is not disturbed when some nearby bond is broken. If these conditions are fulfilled, then I_{abs}, the total absorption, is constant throughout a period of irradiation at constant incident intensity I_0. When several absorbing substances are present simultaneously, the fractional absorption by the reacting component is given by

$$\frac{I_{\text{abs}}, \text{(active)}}{I_{\text{abs}}, \text{(total)}} = \frac{e_R c_R}{e_R c_R + e_P c_P + e_M c_M} \tag{8}$$

where each e and c refers to the extinction coefficient and concentration of each absorbing component, R is the reactant, P is the product, and M includes all other absorbers in the medium. If e_R and e_P are equal and if the product builds up at the same rate as the reactant is consumed, then the denominator is constant throughout the reaction, the total absorption is constant, I_{abs}, (active) is proportional to c_R, and Eq. (7) is valid. In such a treatment the difference between reactant and product is often detectable only by biochemical or biological assay, like the difference between active and inactive virus or between native and denatured protein. If a photochemical process is accompanied by an overall change in the absorption, a test of the first order law requires a correction to Eq. (7) (McLaren, 1951).

There are very few, if any, authentic cases of a true second or higher order dependence of rate on light intensity, of the type expressed by Eq. (9).

$$\text{rate} = kI_0^2. \tag{9}$$

The physical interpretation of such an equation is that two light quanta must strike the absorber almost simultaneously in order to induce reaction (Gibson *et al.*, 1953).

Many photochemical reactions follow Eq. (10), in which n is a

$$\text{rate} = kI_0^n \tag{10}$$

fraction between zero and one. Reactions having this type of behavior are invariably complex, in which the effect of at least one rate-determining dark reaction must be superimposed on the primary light effect. This behavior can be expected where the primary photoproduct is an unstable intermediate and there is a competition between a completing reaction which produces a finished stable product and a bimolecular reaction which destroys the intermediate.

An analogous situation appears in the reversible bleaching of chlorophyll in methanol solutions. Knight and Livingston (1949) observed that the extent of bleaching is proportional to the square root of the light intensity. They explained this aspect of their results by assuming the following mechanism, where HChl, HChl*, and Chl signify normal chlorophyll,

$$\begin{aligned}
&(1)\ \text{HChl} + \text{light} \rightarrow \text{HChl}^* && \text{rate (1)} = I_{\text{abs}}\\
&(2)\ \text{HChl}^* + \text{B} \rightarrow \text{Chl} + \text{BH} && \text{rate (2)} = k_2(\text{HChl}^*)(\text{B})\\
&(3)\ \text{Chl} + \text{BH} \rightarrow \text{HChl} + \text{B} && \text{rate (3)} = k_3(\text{Chl})(\text{BH})
\end{aligned}$$

photo-excited chlorophyll, and a radical resulting from the oxidation of chlorophyll. The observed bleached form of chlorophyll is assumed to be this radical. The oxidizing agent is B, either the solvent or an impurity in the solvent, and BH is the radical resulting from its reduction. The rate of each partial reaction is assumed to follow the law given next to the reaction. At the steady state, the concentration of the radical (Chl) is constant and equal to (BH); also (HChl*) is constant. Thus,

$$\frac{d(\text{HChl}^*)}{dt} = 0 = \text{rate (1)} - \text{rate (2)}$$

$$\frac{d(\text{Chl})}{dk} = 0 = \text{rate (2)} - \text{rate (3)}$$

$$\text{rate (3)} = \text{rate (1)}$$

$$(\text{Chl}) = (\text{BH}) = \left(\frac{I_{\text{abs}}}{k_3}\right)^{\frac{1}{2}}$$

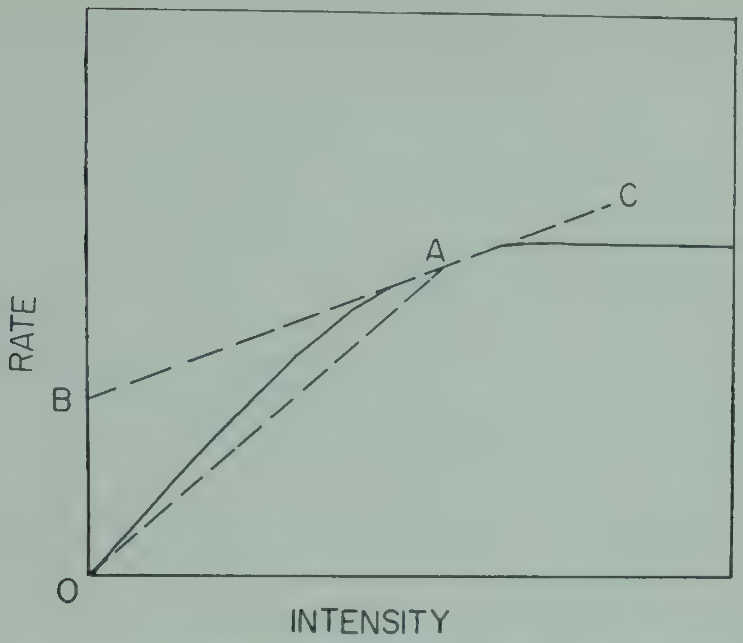

FIG. 3. Generalized saturation curve. The slope of the broken line OA represents the integrated, or average, quantum yield over the range of intensities from zero to the intensity at A. The slope of the broken tangent BAC represents the differential quantum yield at point A. See text for discussion.

Livingston and Ryan (1953) confirmed the second order back reaction of the two radicals by measuring the reversal of the bleaching after a single strong flash illumination. A monograph on photochemical kinetics should be consulted for the methods of evaluating and interpreting more complicated rate data (Noyes and Leighton, 1941; Rollefson and Burton, 1939).

Deviations from the first order law give important information about the complexities of the system. For example, rates which show a linear dependence on light intensity at low light but no dependence at high intensities indicate the presence of a limiting non-photochemical step. This is illustrated in Fig. 3. At low light, the photochemical process limits the overall reaction, but at high light a dark reaction is limiting. Since the rates of dark reactions do not depend on light intensity, a limiting, or saturation, overall rate is reached. Although the participation of a slow dark reaction is easily proved from this type of experimental curve, it is impossible to deduce the nature of the limiting dark reaction by photochemical techniques alone. One possibility is a diffusion process, in which an important substrate must reach the absorber substance before the excitation energy is dissipated. Another possibility is a deficiency in the amount of a substrate or in the rate of replenishing the supply of substrate. If the nature of the limiting substrate is known or suspected, an increased supply of that substance should increase the overall rate. Another possible type of limitation is the amount of an enzyme needed for one of the reactions constituting the overall process. A special type of limiting dark reaction peculiar to photochemical processes may occur if free radicals play a role in the mechanism. Radicals are often the primary photochemical products resulting from the breaking of a bond. In gaseous reactions such primary radicals quickly separate from each other, but in liquids or solids the radicals are hemmed in by the enveloping molecules of the environment. This phenomenon is known as the Franck-Rabinowitch "cage" effect (Franck and Rabinowitch, 1934). There is consequently a high probability of recombination of the radicals during the many collisions they may have before escaping from the "cage." In order to avoid these back reactions, which would undo the effect of the light, a fast dark reaction may be necessary to stabilize one or both of the radicals. Such stabilizing events have indeed been postulated to occur in photosynthesis, and the normal saturation limit of photosynthesis in steady light is believed to be related to the finite amount of the enzyme which catalyzes this reaction (Franck and Herzfeld, 1941).

(2) *Quantum yield.* Shortly after the quantum nature of light was recognized, Einstein proposed a law of photochemical equivalence, according to which the photoactivation of one molecule or atom requires the absorption of one quantum of light. This statement does not necessarily mean that a stable reaction product must arise from each act of light absorption. Because of the many alternate paths for the electronic excitation energy it is only the exceptional case in which each absorbed quantum produces a chemical effect. The Einstein law was intended to be a statement of the non-divisibility of the light quantum; that is, a chemical reaction, when it does occur, is triggered as the result of the absorption of a whole light quantum, not a half or a third of a quantum. Nevertheless, the Einstein law makes no restrictions on the possibility of a sequence of photochemical reactions. For example, the photosynthetic production by green plants of one molecule of oxygen would require the absorption of at least three light quanta, even if the light energy were converted completely to chemical energy. It is well known that photosynthesis is a complicated sequence of events, and indeed some of the intermediates in the conversion of carbon dioxide to carbohydrate have been identified. It is therefore to be expected that several of the component steps may require light energy; and it is not at all necessary to assume, as some have done, that the entire conversion of carbon dioxide to carbohydrate must be triggered by one quantum in order to conform with the Einstein law of photochemical equivalence.

The actual efficiency with which absorbed light is utilized for chemical purposes is called the quantum yield, ϕ, where

$$\phi = \frac{\text{number of molecules of product}}{\text{number of quanta absorbed}} \tag{11}$$

$$\phi = \frac{\text{number of moles of product}}{\text{number of einsteins absorbed}} \tag{12}$$

The value of ϕ would be 1 if each photo-excited molecule produces a molecule of finished product, greater than 1 if the light initiates a chain reaction, and less than 1 if a series of photochemical steps is involved or if some of the absorbing molecules lose their energy of excitation by one of the non-chemical processes discussed in Section I.2. It is clear from the defining equation that ϕ is independent of light intensity for a reaction that is first order with respect to light. For those complicated biological systems in which the order is not constant it is most meaningful to determine ϕ under conditions where the reaction is most nearly first order. In the case represented by Fig. 3, for example, ϕ is proportional to the slope of the curve. The proportionality constant would be 1 if the rates were expressed in moles of product per second and the intensity in einsteins per second times the fractional absorption. At point A the curve no longer has its original slope and ϕ no longer has its maximum value. There is some confusion in the literature about the significance of ϕ at such a point. A single determination of the reaction rates at A would have led to the conclusion that ϕ is the slope of the straight line from the origin, O, to A. But observational knowledge of the entire curve suggests that a differential value of ϕ pertaining to this particular light intensity is better given by the slope of the curve represented in the figure by the tangent at point A, line BAC. This difficulty points up the desirability of determining ϕ in the linear region. Sometimes, however, there is good biological reason for suspecting different types of reaction at different intensities. In photosynthesis, for example, the respiration at low light intensities might not be the same as at high light. Another example is the transition at very high light from useful photosensitized reactions to damaging photo-oxidations. For such cases there is a real advantage in studying ϕ in a non-linear region. The choice of the method of measuring the slope must be a matter of judicious analysis for each individual problem.

The experimental determination of ϕ requires two types of measurements, a chemical or biological assay for the extent of reaction and a physical observation of the amount of light absorbed. Little need be said here about the first quantity except for the special problems of assay related to photochemistry. The expression of the extent of reaction on a molecular or molar basis sometimes presents problems that do not usually confront the biologist. If the reaction is the inactivation of an enzyme, some arbitrary unit of activity is sufficient for most applications; but the molecular weight of the enzyme must be known for evaluation of the quantum yield from Eq. (12). Of course if the inactivation is definitely associated with a particular chemical reaction, the disappearance of —SH groups for example, then an assay for this particular group is a sufficient molecular basis for substitution in Eq. (12). Another problem of assay that is peculiar to photochemical measurements is design of the reaction vessel. The reaction must be followed under circumstances of known light intensity and light absorption. Consequently some ingenuity is required in design and assembly of the equipment so that the photometric measurements may be applicable to the reaction as it is analyzed. Some of the correlation methods that have been used will be discussed in the following treatment of the light measurements.

The incident intensity is usually measured by removing the reaction cell and placing a light detector in exactly the same position as the front window of the cell. Usually, the intensity of the incident light, I_0, and the fractional absorption, F, are measured separately. The intensity of absorbed light is of course the product of these two quantities. The fractional absorption is given by Eq. (13). If the optical density, D, is sufficiently

$$F = 1 - 10^{-D} \tag{13}$$

great (above 1.5), the absorption is practically 100% and need not be measured. For solutions or suspensions that are less concentrated, the optical density can be measured in any spectrophotometer and related to the density in the photochemical cell by Lambert's law,

$$\frac{D_1}{D_2} = \frac{l_1}{l_2} \tag{14}$$

where D_1 and D_2 are the optical densities in the spectrophotometer and reaction cells and l_1 and l_2 are the respective lengths of the optical paths in the two cells. The measurement is most unambiguous if both cells have plane parallel windows and the solution completely fills the space between the windows. (If the reaction vessel is a manometer vessel which has a shaken gas-liquid interface, the length of the optical path is uncertain on account of the shaking.) A value of F determined this way represents the fractional absorption of the light falling on the cell. If the cross-section of the incident light beam does not fall completely within the cross-section of the cell, then there will obviously be zero absorption for part of the beam. An error would result if the incident intensity were measured for the whole beam and the fractional absorption for only part of the beam. The method can therefore be applied only if the incident intensity is measured over an area no greater than the area of the absorbing solutions. For larger beams there are several methods for evaluating just that portion of the incident intensity which strikes the reaction cell. The first method is to measure the intensity actinometrically with the reaction vessel itself serving as the actinometer vessel. The cell must of course be in the same position for the actinometric measurement as for the reaction itself. The special advantage of this method is the ease of interchange of the reaction solution or suspension with the actinometric solution. If another method is used for the intensity measurement, a screen can be made having an opening exactly the size of the front window of the reaction cell. This screen, if placed in the same position as the reaction cell front window, will admit to the light detector the same amount of light as would enter the reaction cell. Still another method can be used if the light beam is known to be perfectly uniform and parallel. In this case the intensity per square centimeter times the area of the front window of the reaction cell gives the intensity incident on the cell.

A more general method is to determine the transmission directly in the reaction cell by measuring the intensity of light transmitted through the rear window when the cell is filled with the sample and also when the cell is filled with the suspending medium alone. The difference between the two intensities represents the absorption by the reactants. As long as the detector receives all the light from the beam there are no restrictions on the relative cross-sections of the beam and the cell or on the shape of the cell. Of course the greatest sensitivity will be achieved if most of the light beam passes through the cell.

In the above discussions we have assumed that light is either absorbed or transmitted by the reaction components. This assumption fails if there is light scattering. In such a case light would leave the reaction cell at the sides and even at the front as well as at the rear. The true absorption would no longer be equal to the difference between the incident and transmitted intensities. The principal cases of scattering are scattering by high molecular weight substances in solution and scattering at phase boundaries such as those occurring in suspensions, in cells or tissues, or in solids. The three methods of solving these problems are to calculate, to measure, or to avoid the effects of scattered light. Not all three methods are possible in any one case, as will be seen from the discussion below. A third type of scattering, fluorescence, occurs as a result of absorption, not as an alternate to it. If desired, the number of quanta lost from possible chemical reaction by this route can be subtracted from the total absorption after an independent fluorescence measurement is made.

If the sample is a true solution and if the dissolved molecules are small compared with the wave length of light, the intensity of scattered light can be calculated from the well-known equations for scattering. Alternately, the scattering of the same sample could be measured at a series of wave lengths at which true absorption does not occur. These values could be extrapolated to the particular wave length of the photochemical experiment to evaluate the scattering correction. The equations and the methods for determining scattering are described in Chapter II by Oster. If the scattering cannot be evaluated directly, sometimes an approximation can be made by a non-experimental calculation of the true absorption. Oster and McLaren (1950) studied the ultraviolet inactivation of tobacco mosaic virus. They measured, from the per cent transmission of the light beam, the sum of the true absorption and the scattering. Recognizing that scattering removed about as much light from the beam as did true absorption, they estimated the true absorption in terms of the known content of chromophoric groups, nucleic acid, tryptophane, tyrosine, and phenylalanine in the virus. They assumed that each of these groups possesses the same molar extinction coefficient in the virus as it has alone. The obvious pitfalls of applying this method generally are the minor uncertainty about the constancy of the extinction coefficient and the major uncertainty that all the important absorbing groups have been identified and analyzed. If any of the above methods is used to evaluate the scattering, then the true light absorption, for use in Eq. (12), is the amount of incident light minus the sum of the transmitted and scattered light.

It is sometimes possible to arrange the apparatus for the photometric measurement so that the light detector catches all light leaving the reaction cell not only in the forward direction of the beam but at all angles with respect to the incident beam. Such arrangements are called light integrating devices. If the integrating detector measures all light which leaves the reaction cell at any angle, then the true absorption is simply the incident intensity minus the integrated transmitted intensity. (The integrator may or may not measure fluorescence depending on whether the light detector is sensitive to the wave length of the fluorescence as well as to the incident wave length.)

A simple, but crude, integrator is a large area detector placed close to the reaction cell. Emerson and Lewis (1942) constructed a narrow absorption cell with sides made of brass plated on the inside with nickel so as to reflect the scattered light back into the suspension. Most of the light leaving the cell then left at the rear window. A large area photronic cell was placed within a millimeter of the rear window so that most of the emerging light was intercepted.

Brackett *et al.* (1953) employed a mechanical integrating procedure. They mounted a light detector on a carriage that could be so rotated that the light intensity could be measured at many points on a cylindrical surface concentric to the vertical axis of the reaction cell. By plotting the intensity as a function of position on the cylindrical surface they were able to calculate the total light intensity transmitted and scattered by the sample by a process of numerical integration. The ratio of two such integrated intensities, one with the reaction suspension in the cell and one with a blank solution in the cell, gives (1-F) directly, where F is the fractional absorption. This method is admittedly a tedious one, and was used as a last resort because of the unsuitability of other methods for the particular problem.

An ingenious device for integrating the scattered with the transmitted light was used by Warburg *et al.* (1951). They used a chemical actinometer as the detector, and they built the actinometer vessel completely around the reaction vessel, leaving exposed only a small area of the latter through which the light beam entered. They estimated that the light back scattered through the entrance way was not more than 1% of the total light. All other light was either consumed by true absorption in the reaction vessel or was captured and measured in the actinometer.

Several integrators have been reported which utilize reflecting surfaces. Noddack and Eichhoff (1939) constructed a hollow ellipsoid of revolution large enough to accommodate within it the biological sample and the light detector. The inside of the ellipsoid was a mirrored surface. The sample was placed at one of the elliptical foci and was illuminated by a beam of light entering from the outside through a small window along the axis of revolution. The detector was placed at the second focus and was designed so as to respond to light striking it from all angles. The detector is thus placed so as to receive not only the directly transmitted beam, but also all the light scattered by the sample and reflected from the ellipsoidal surface. The geometrical principle is that light originating at one focus of an ellipsoid, regardless of angle, will be reflected, after one surface reflection, upon the second focus. If suitable corrections are made for deviations of the mirror from perfect reflectivity and for the small amount of back-scattered light lost through the entrance window, the method gives reliable values for the true absorption. The original reference should be consulted for further details. A survey of other types of integrators, including a spherical mirror, is given by Mestre (1935).

The most common type of integrator is the Ulbricht sphere, an instrument that has long been used in optical laboratories for measuring the total radiant output of light sources. The sphere is coated on the inside with a white paint that is ideally a perfectly reflecting surface. The paint is applied to give a mat finish so that the reflected light is diffuse. The principle of operation of the sphere is that any portion of the surface acquires a brightness which is proportional to the total amount of radiation striking the entire surface. Since the rays of light are repeatedly reflected from the surface at random angles, the distribution of radiation throughout the sphere is uniform. In operation, the sample is mounted inside the sphere so as to receive the incident beam coming from the outside through a window. All light that is transmitted or scattered by the sample will reach the spherical surface. The light detector, shielded from light that might come directly from the sample without first reaching the walls, could be an opal glass window in the sphere whose brightness could be determined by photometric measurements made on the outside. In current practice the detector is usually a photoelectric cell mounted inside the sphere with its sensitive surface facing a section of the spherical surface that receives illumination only indirectly. Ideally, (1-F) should equal the ratio of the response of the detector when the sample is in place, D_s, to the response, D_0, when a blank is in place. In practice, imperfections in the sphere, nonuniformity in the distribution of scattered light, light loss through the entrance window, and other deviations from ideality are compensated by comparing the response of the detector with two fixed points. One fixed point, D_R, is determined by filling the reaction cell with a suspension of a perfectly reflecting substance, like calcium carbonate, of approximately the same turbidity as the sample. The other fixed point, D_A, is determined by filling the reaction cell with India ink, a perfect absorber. Then,

$$F = \frac{D_R - D_S}{D_R - D_A}, \tag{15}$$

Applications of the method to biological problems have been described by French and Rabideau (1945), Rieke (1949), Vermeulen *et al.* (1937), Forster and Livingston (1952), and Warburg (1953).

The previous discussion of absorption has been devoted to the experimental determination of the quantity, the amount of light absorbed. This quantity, when converted to a quantum basis, can be substituted in the denominator of Eq. (12) for calculation of the quantum yield. Sometimes there may be some reason for basing the efficiency of a reaction not on the total absorption of the sample but on the number of quanta absorbed by one component or group of components in the sample. In the photochemical destruction of the pyrimidine related to thiamine, for example, the reaction product, although inactive with respect to a biological assay, absorbs light about as effectively as the active pyrimidine (Uber and Verbrugge, 1940). Quantum yields were therefore calculated by correcting the light absorption to the amount absorbed by the original pyrimidine. In the photochemical denaturation of proteins both the native and denatured protein may absorb light to the same extent. Only the native protein, however, is capable of undergoing a reaction which is experimentally demonstrable. Many workers have therefore reported quantum yields in terms of the amount of light absorbed by native protein (McLaren and Pearson, 1949). The total amount of light absorbed must be multiplied by an average value of the fraction of the protein that was native during the duration of the experiment. If this fraction does not change by too large an amount during the course of the measurement, a geometrical average may be used. For larger changes, the correct average amount of active protein, c_{av}, should be evaluated from Eq. (16),

$$c_{av} = \frac{1}{t}\int_0^t c_t\,dt \tag{16}$$

where c_t is the amount of active material at any time, t. The fraction of active protein at any time is c_t/c_0, and for the case of a first order reaction, the average value of this fraction during an experiment of duration t is given by Eq. (17),

$$(c/c_0)_{av} = \frac{(c_f/c_0) - 1}{2.3 \log c_f/c_0} \tag{17}$$

where c_f is the amount of native protein at the end of the experiment. Another form of this equation has been used by McLaren and Pearson (1949).

The value of the quantum yield is important because it may give a clue to important structural features of biological systems. A quantum yield of one can be interpreted very simply to mean that every absorbed light quantum is effective for the chemical or biological reaction. A quantum yield of less than one does not have so simple an interpretation. A poor yield may mean that most of the absorbed energy is immediately dissipated as heat or fluorescence; or that photochemical reactions other than the one being analyzed are occurring; or that the main reaction is occurring with good yield but the reaction quickly reverses in the dark; or that a primary photochemical intermediate, perhaps a free radical, undergoes destruction by a side-reaction with a protective substance instead of leading to a well recognized end product. Comparison of the quantum yields for different substances often provides a suggestion as to the reason for poor photochemical efficiencies. Oster and McLaren (1950) have observed that the quantum yield for inactivation of tobacco mosaic virus by ultraviolet light is about 500 to 100 times smaller than the quantum yield for the rupture of disulfide bonds or peptide bonds in simple model substances. From this they draw a possible conclusion that only one in every 500 or so absorbing aromatic groups in the virus is a "sensitive" spot. The general trend of decreasing quantum yield for protein inactivation with increasing molecular weight is an experimental fact that may have several possible interpretations (McLaren *et al.*, 1953). One of these is the "sensitive spot" hypothesis.

According to this, only a small number of chromophoric groups are strategically situated with respect to the architectural features of the molecule governing its activity. All other chromophoric groups dilute the overall effectiveness of the radiation by absorbing light at sites far removed from the important functioning points of the molecule. Another possibility is that at the "sensitive spot," by reason of some special environmental factors that might include hydrogen bonding or bond strains, the probability of bond breakage is greater than at an average spot even though the chromophoric groups may be the same. Another possibility is that big molecules, by virtue of their sheer size, can protect themselves from the damaging effects of radiation. A plausible mechanism for self-protection is the recombination of free radicals. The cage effect, discussed above in Section I.3.*a*. (1), could be enhanced at increasing molecular size because the fragments resulting from a photochemical dissociation will find it increasingly difficult to escape from the framework of the increasingly complex intra-molecular environment. It is not the intention here to attempt to evaluate these and other hypotheses, but merely to point out the fruitful ideas about structure of complicated molecules that can be gained from photochemical studies.

McLaren *et al.* (1953) have used quantum yield measurements as the basis for other conclusions about protein activity. The fact that certain relatively small proteins have a quantum yield for inactivation greater than the known quantum yield for splitting disulfide or peptide bonds led these investigators to the view that the inactivation process, at least in the photochemical case, may involve an attack on the aromatic groups in the protein rather than an attack on the polypeptide chain itself. In this same paper, another observation was made that has some important structural implications. The quantum yield was studied for the liberation of alanine from a series of compounds having the general formula $C_6H_5(CH_2)_nCO$ $NH\ CH(CH_3)COOH$. When n was 1, 2, or 4, the quantum yield was almost the same, about .004. When n was 3, the quantum yield was .055. This peculiar sensitivity of the phenylbutyrylalanine was tentatively interpreted in terms of the folding of the molecule about the polymethylene chain so that the amide bond can come closest to the chromophoric phenyl group when n is 3. pH dependence of the quantum yield for inactivation of proteins has been another type of photochemical observation that has been correlated with structure. Many proteins show a minimum value of the yield near the iso-electric point (McLaren, 1949). The interpretation has been advanced that since the protein is most compact at the iso-electric point, the recombination of radicals by the caging effect would be greatest. On either side of the iso-electric point the molecule will be stretched out because of repulsion of like charges; hence, radicals formed by a primary light process will have a greater chance of escaping from each other before recombination could occur.

In most of the common photochemical reactions, one quantum of light has a finite probability of triggering the entire reaction. The fact that the observed quantum yields are less than one generally means that other processes are competing with photochemistry for the energy of excitation. There is an important category of reactions in which a different situation arises. These are the step-wise processes involving more than one primary photochemical step. In such cases, the quantum yield has to be less than one even if there is no wastage of the energy of excitation by competitive processes. A simple example is the chlorophyll-sensitized oxidation of phenylhydrazine by methyl red. Livingston and Pariser (1948) found that the maximum quantum yield for this reaction is about 0.5. They interpreted their results on the assumption that one quantum is necessary for the transfer of one hydrogen atom from the phenylhydrazine to the methyl red. Since the overall reaction requires a transfer of two hydrogen atoms, the quantum yield for the whole process cannot be greater than 0.5.

A similar situation occurs in photosynthesis, and the reason is a very simple one. The reaction of one molecule of carbon dioxide with water to give one molecule of oxygen plus carbohydrate is an endothermic reaction requiring more energy than is contained in a single quantum of light absorbed by chlorophyll. On a molar basis, about 115 kcal is needed for the reaction and the amount available in light of 7000 A, the longest wave length which chlorophyll can utilize efficiently, is only 41 kcal per einstein from Eq. (2). Thus, the maximum quantum yield would be $^{41}/_{115}$ or .36 if all of the light energy were converted completely into the chemical energy for the overall reaction. If the production of one molecule of oxygen requires an integral number of photochemical steps, then the quantum yield would be .33 for a three-step process, .25 for a four-step process, and so on. This calculation is independent of the mechanism of the reaction. An experimental determination of the quantum yield should be expected to put an upper limit on the number of successive photochemical steps in photosynthesis. In fact it is this motivation which has prompted so many investigators to measure the quantum yield under optimum conditions. A definitive knowledge of the number of steps would be of great assistance in proposing and evaluating various possible mechanisms for the photosynthetic process. Unfortunately the desired numerical value is very difficult to determine, and universal agreement has not been reached among the active workers in this field. The chief difficulty is the sorting out of the photosynthetic reaction from the many concurrent metabolic reactions occurring in the green plant cells at the time of the measurement. Although the details of the experimental investigations and of the problems of interpreting the relative contributions of photosynthesis and respiration are beyond the scope of the present discussion, some brief mention will be given here of the relevancy of the general principles of photochemistry to this problem.

The complete conversion of light energy into chemical energy of complicated molecules is as improbable as the conversion of the heat of the oceans into the work of driving ships. Losses of whole quanta may not be important, since only about one excited chlorophyll molecule in a thousand loses energy by fluorescence and since complete vibrational dissipation might be avoided in the presence of an efficient photochemical mechanism. Also, losses by back reactions could be extremely low if the pigment-enzyme complex in the chloroplasts serves to steer the products from one step into the machinery for the next step. The important energy losses that cannot be avoided have to do with the distribution of the energy of a single quantum, partly into the chemical energy of the reaction and partly into heat. These losses have been evaluated critically by Franck (1953).

One reason for inefficiency, common to all photochemical reactions, is that the chlorophyll molecule, at the instant that it transfers excitation energy to some acceptor system, is left not in its lowest possible energy state but in a state corresponding to vibrational excitation. This was discussed in Section I.2.*a*. in connection with the red shift of fluorescence spectra. The vibrational excitation remaining in the chlorophyll after the act of chemical sensitization is similar in kind to the excitation remaining after fluorescence. This energy of vibration, amounting to a few kilocalories per mole, will be transferred to the medium as heat by impacts. Another source of energy loss would occur if chlorophyll entered into its sensitizing reaction by way of a phosphorescent state rather than the directly excited fluorescent state. There is indirect evidence (see Section II.2.) that some form of metastable state of chlorophyll is involved in sensitized reactions. If so, some conversion of energy into heat must accompany the internal conversion from the fluorescent state to the metastable state. This loss may be thought of as the stabilizing energy for the metastable state. It is represented in Fig. 1 as ΔE. If the metastable state did not represent a partially de-energized condition, then it could transform spontaneously to the fluorescent state, from which all the energy would be lost as fluorescence. A third type of loss peculiar to photochemical reactions is the excess kinetic energy that must be imparted to the products of the primary dissociation so that the resultant fragments will separate from each other and not recombine. In this, as well as in the two previous types of energy dissipation, the losses occur by the endowment of the medium with excess vibrational energy, that is, heat. This type of energy is transferred in such small quantities that it is very improbable that it can be recollected in any one spot in an amount sufficient to rupture a chemical bond.

That the overall energy of the reaction is not the single factor determining the quantum yield of such reactions is illustrated by the study of other systems. One simple system investigated in some purple sulfur bacteria is the reduction of carbon dioxide with hydrogen sulfide. The photosensitizing agent is bacterio-chlorophyll. When the products are carbohydrate and sulfur, the net energy requirement of the reaction is close to zero. Yet the quantum yield is the same as for green cell photosynthesis. Certain green algae, after anaerobic adaptation, can perform a photochemical reduction of carbon dioxide with molecular hydrogen. The products are carbohydrate and water. Although this reaction is exothermic, the same number of quanta are required per carbon dioxide molecule as in normal photosynthesis (Rieke, 1949). These experiments have been taken to suggest that the photochemical steps are the same in all cases. The mechanism of hydrogen transfer, rather than the energy requirement of the overall process, apparently determines how many quanta should be required.

b. Wave Length Dependence

There are many photobiological reactions that depend only on the total number of quanta absorbed irrespective of wave length. This is true of most of the photo-sensitized reactions because the sensitizer molecule is quickly brought to the lowest vibrational level of the fluorescent state by internal conversion regardless of the wave length of excitation. This phenomenon is related to the constancy of the fluorescence spectrum discussed in Section I.2.*a*. In fact, the constancy of the quantum yield with wave length is so typical that deviations are generally taken as positive proof of some complicating factor. Emerson and Lewis (1943) found that the quantum yield for photosynthesis in *Chlorella* was independent of wave length in the red, where all light absorption is due to chlorophyll. At 4800 A, however, the quantum yield had dropped by 30%. At this wave length, carotenoids are the chief absorbers. The conclusion was that the quantum yield for each absorbing pigment is a constant, but that different pigments may have different efficiencies. In a similar manner, Milatz and Manten (1953) found that the quantum yield for phototaxis in *Rhodospirillum rubrum* was constant over a wide wave length range as long as the absorption was confined chiefly to the bacterio-chlorophyll. At the wave length at which the carotenoid spirilloxanthin absorbed strongly, the quantum yield dropped to a lower value. Such observations as these have been used to determine the quantitative relative efficiencies of different pigments.

This type of phenomenon has been observed for simpler systems containing only one type of absorbing molecule. Setlow and Doyle (1953) have found that the quantum yield for the photoinactivation of chymotrypsin increases very slowly with decreasing wave length from 2950 A to 2537 A, but increases very rapidly below 2400 A. They attribute this behavior to excitations in different regions of the protein. Above 2400 A the principal absorptions are those of the aromatic groups; below 2400 A the absorption of the peptide linkage becomes important. If this explanation is correct, it is of course possible that the chemical nature of the inactivation may not be the same in the two cases. A general pitfall about work in the far ultraviolet, about which photobiologists should be warned, is the possibility of photochemical formation of ozone from oxygen below 2500 A. The indirect effects of this reaction can be avoided by performing irradiations in this region of the spectrum anaerobically.

It is not necessary that a variation of the quantum yield with wave length is proof of a change in the type of electronic excitation. For direct, nonsensitized, photochemical reactions, there is no good theoretical reason why the quantum yield should be a constant. Forbes (1935) has shown that the quantum yield for the photochemical oxidation of quinine by chromic acid varies considerably as the wave length is changed within a single absorption band of the quinine. Zimmerman (1949) showed that the quantum yield for the photochemical oxidation of water by potassium permanganate increases continuously with decreasing wave length within a single absorption band of the permanganate ion. In fact, this behavior is almost a general phenomenon where a bond is ruptured in the absorbing molecule. This can be explained by a model similar to the Rice-Ramsperger-Kassel model for unimolecular reactions (Kassel, 1932). As the wave length of excitation is lowered, the absorbing molecule is endowed with a surplus of vibrational energy. If reaction occurs when a certain critical amount of vibrational energy is concentrated in a particular bond, then a finite time is required for the vibrational energy to be accumulated in that bond. Before the crucial accumulation occurs, the energy may be regarded as fluctuating more or less at random among various neighboring bonds. During this period of vibrational fluctuation the molecule also is in constant danger of losing its vibrational energy by impact with the surrounding medium. The greater the surplus of vibrational energy at the instant after the absorption of light, the less will be the time required for accumulation of the critical amount of energy in the critical bond, and the less will be the chance of a de-activating collision. This model has been successful in accounting for the observed kinetics of many dark unimolecular reactions and probably is fairly reliable for treating some photochemically activated molecules. One of the weak points of this argument is that the fluctuation of vibrational energy through the various bonds is regarded as random. It is possible to conceive of cases where the probability for migration of vibrational energy from one bond to another is a different function of energy for each pair of bonds. If so, some cases might be observed in which the quantum yield is not a uniformly increasing function of the light frequency.

c. Concentration Dependence

The simplest role of concentration in a photochemical reaction is to determine the amount of light absorbed from a given incident beam. Because most photochemical reactions are a complicated sequence of dark and light reactions, the overall rate is usually dependent, in more subtle ways, on the concentrations of absorber, inhibitor, catalyst, or reaction partner. The problems of sorting out the various concentration factors in a photochemical reaction are the same problems that exist in general reaction kinetics. The reward of a careful study of the concentration effects is equally great in light and dark reactions, namely, a more detailed insight into the mechanism of the reaction. In some respects, photochemical kinetics is simpler because the nature of the "activated state" is better known. Thermal excitations of molecules are statistical in nature in that they produce molecules in many different high energy states. Photo-excitation, on the other hand, produces molecules in a definite, known state of excitation. For this reason, photochemistry has often been used as a tool for studying reaction mechanisms, even when there is no practical interest in light effects.

The key point about photochemical kinetics is that the excited molecule must undergo chemical reaction quickly or it will lose its energy of excitation by fluorescence, phosphorescence, or internal conversion. Concentrations enter into the balance sheet in changing the competitive positions of the chemical versus the non-chemical methods of utilizing the excitation energy. In the photolysis of riboflavin and its analogs, for example, Halwer (1951) has shown a generalized acid-base catalysis, the overall rate being dependent on the concentration of the added catalyst. The role of the catalyst, according to his view, is to facilitate an internal proton transfer from a hydroxyl group on the ribose side chain to an isoalloxazine nitrogen. In the absence of the catalyst, this transfer would be slow compared with internal conversion.

The competion of substrate for a photoexcited molecule often follows the well-known quantitative law,

$$\text{rate} = \frac{a(X)}{1 + b(X)}, \tag{18}$$

where a and b are constants and (X) is the concentration of some reactant. This law can be derived by assuming that X reacts with the excited molecule by a second-order reaction (rate is proportional to the concentration of excited molecules times the concentration of X) and that the excited molecule can lose its excitation energy by non-chemical dissipative processes which are all first order. The constants a and b are related to the rate constants for the chemical and non-chemical processes. At high concentrations of X, the rate approaches a limiting value independent of (X). This means that every molecule that reaches the excited state will find an X molecule and will react. The rate is then dependent only on the rate of excitation. At very low values of (X) there is not enough X to go around for every excited molecule, and the rate is directly proportional to (X). This equation has been found to apply to both the oxidizing and reducing agents in the chlorophyll-sensitized oxidation of phenylhydrazine by methyl red (Livingston and Pariser, 1948) and to the concentration of ascorbic acid in the photobleaching of Rose bengal (Oster, 1953).

d. Temperature Dependence

Purely photochemical steps have practically no temperature coefficient because the excitation is provided by light instead of thermal agitation as in dark reactions. Warburg and Negelein (1928), for example, measured the quantum yield for the photodissociation of carbon monoxide from carbon monxy-ferrocystein and from carbon monoxy-pyridine hemochromogen over a wide range and found practically no variation. If a photochemical reaction rate is found to have a temperature coefficient, this is direct evidence for the occurrence of at least one dark step in the sequence.

e. Intermittency Dependence

The use of flashing light is a technique that can sometimes be used to study the separate light and dark steps of a photochemical sequence. If a reaction is truly first order with respect to the light intensity, there should be no difference in the extent of reaction whether a given amount of radiation is given as a steady low intensity dose, as a steady high intensity dose over a shorter period, or in a series of short bursts. If the reaction is limited partly by a dark process, there could be a dependence on the manner in which the radiation is administered. Emerson and Arnold (1932) were the first to take advantage of this fact in studying the dark reactions of photosynthesis. By use of a rotating sector which interrupted the incident light beam, they provided their samples with alternating periods of light and dark. They found that for light flashes of a constant intensity and duration the yield of reaction product per flash could be increased if the period of darkness between flashes were prolonged. Apparently the dark time was being used either for the formation of substrates for a succeeding flash or for the stabilization of labile products of the previous flash. As the dark time was increased further, a saturation effect occurred and no further improvement of the yield could be obtained. Apparently enough time had already been allowed for the dark reaction to go to completion. This technique thus provided a direct measure of the time required for the dark reaction and also of the amount of "dark factor" which placed an upper limit on the yield per flash. In addition, this method made it possible to study the effect on the dark reaction alone of external variables like temperature, inhibitors, and concentrations.

Franck (1949a) has discussed a curious type of intermittency effect resulting from the unequal illumination of different parts of the reaction vessel. This can happen if the incident beam is smaller in cross section than the cell window, or in the case of large beams if the percentage absorption is so high that the rear portion of the cell is at a much lower intensity than the front. He has pointed out that rapid stirring of a sample is not sufficient to guarantee that any one molecule will give the same effect as if illuminated continuously at the average intensity. Rather, a type of flashing will occur as any one molecule is moved from regions of darkness in the cell to regions of relatively greater brightness. This complication should be avoided whereever the possibility of an intermittency effect is suspected.

The rotating sector can be used for studying dark reactions as fast as a thousandth of a second. For studies of dark reactions faster than this the single flash technique can sometimes be used. For this method a short flash of light is used and the subsequent dark reaction is studied by a rapidly responding analytical procedure. Livingston and Ryan (1953) were able to determine by this method that the half-life for the dark reversal of the photobleaching of chlorophyll in methyl alcohol is 5×10^{-4} seconds. They measured the reappearance of the normal chlorophyll spectrophotometrically, and recorded the light transmission of the sample on a cathode ray oscillograph. A particular advantage of the single flash technique is that very great instantaneous light intensities, that could not be maintained continuously, are possible in a single flash. Thus, the chlorophyll was 100% bleached by the single flash, but the extent of bleaching normally observed under strong steady light is less than 1%.

II. Some Applications of Luminescence

1. FLUORIMETRY IN ANALYSIS

Fluorescence has been used for both the qualitative and quantitative analysis of important biochemicals. In some cases it has a sensitivity far exceeding absorption photometry. This is because a given intensity of fluorescence may be a very large percentage increase over the blank background of a non-fluorescing substance, while an absorption of the same intensity represents a very small percentage decrease with respect to the incident intensity. The limits of detection of quinine sulfate, thiamine, and riboflavin by fluorimetry are all about .001 μg per ml (Loofbourow, 1943). Some substances, like quinine, riboflavin (Cohen, 1935), and chlorophyll, can be analyzed by virtue of their own fluorescence; and others, like thiamine, can be converted to fluorescent products by specific chemical procedures (Jansen, 1936). Details of assay techniques have been worked out in many cases, and references to the original works may be found in various review articles and monographs (Emmett *et al.*, 1941; Hennessy, 1941; Pringsheim, 1949).

In very dilute solutions the fluorescence intensity is proportional to the concentration. This proportionality is obeyed only to the extent that the absorbed intensity is proportional to the concentration, that is, for solutions transmitting at least 90 % of the absorbed light. A calibration curve can always be constructed empirically for the analysis of a given substance in a particular instrument over wide ranges of concentration.

The reason that fluorescence analysis is not used more widely is that the simple behavior outlined in the preceding paragraph is modified in actual cases by several disturbing factors. The most significant of these factors is quenching, the reduction of the fluorescent intensity by small amounts of foreign substances. Oxygen, unfortunately, is one of the most common quenchers. Since the theoretical interpretation of quenching is incomplete, it is impossible to predict what substances might act as quenchers (Livingston and Ke, 1950). From the analytical point of view, this means that the solution for fluorescence analysis must be of high purity. In some cases this criterion can be achieved by extracting the fluorescent substance from a multicomponent solution by a specific extraction procedure. The reason that fluorescence is more susceptible to foreign substances than is light absorption is that the latter changes only if a permanent complex results from the interaction of the absorber with the impurity. Fluorescence can be affected even if the interaction between the two substances is a very temporary one occurring during the 10^{-8} seconds required on the average for an excited molecule to fluoresce.

Fluorescence intensity is very dependent on the nature of the solvent. Livingston *et al.* (1949) reported that the fluorescence intensity of chlorophyll dissolved in dry benzene is less than 3 % of the intensity observed if .01 % water is added to the benzene. Fluoresence is also dependent on temperature, generally decreasing with rising temperature. An additional complication is that in pure solutions most fluorescent organic substances quench themselves at sufficiently high concentrations. This phenomenon of self-quenching is very interesting from the point of view of studying methods of energy transfer between molecules (Watson and Livingston, 1950), but it is a nuisance for the analyst. A consequence of self-quenching is that a plot of the fluorescence against concentration goes through a maximum, so that the analyst must know not only the form of a calibration curve but also on which side of the maximum his solution lies. The maximum usually occurs at concentrations above 10^{-3} molar.

Qualitative fluorescence analysis has found useful applications in recognizing zones in chromatographic separations. Visual observation of fluorescent zones excited by ultraviolet is sometimes possible when the adsorbed substances are too dilute to show color by transmitted light. Fluorescence spectra can be used as a method of characterizing some substances, but cognizance should be taken of solvent effects on the spectrum as well as on the intensity. As a result of sensitized fluorescence the emission spectrum of only one component will often be observed in a solution containing several substances which would fluoresce individually. The one fluorescing substance in a mixture is not necessarily the component present in largest amount, and indeed it may have such a small concentration that its presence cannot be detected by ordinary methods. The component of a mixture that fluoresces most strongly is determined on the basis of the wave length of its fluorescence rather than its relative concentration.

The problems of measuring a fluorescence spectrum are more complicated than the determination of the fluorescent intensity on account of reabsorption. There will always be some overlap between the absorption spectrum of a substance and its fluorescence spectrum, although most of the fluorescence will extend to longer wave lengths. In the region of overlap there is a chance that the fluorescent light will be absorbed before it can leave the solution. This problem can be avoided in simple fluorescent intensity measurements by making the observations at a longer wave length, at which overlap does not occur. The overlap region cannot be omitted, however, if the entire fluorescence spectrum is to be measured. The extent of reabsorption can be made negligible if the measurement is made in very dilute solution. If the measurement must be made in more concentrated solutions, the distortion of the spectrum on the low wave length side caused by reabsorption can be compensated by a complicated experimental and mathematical analysis (Förster, 1951; Duysens, 1952).

2. Fluorescence as an Energy Flow-Meter

Fluorescence, a property of the excited state of molecules, is one of the few tools for studying what happens to excitation energy in the very crucial short period of time in which primary photochemical events take place. A few of the applications of fluorescence to the study of energy transfer processes will be given here.

A quencher lowers the intensity of fluorescence by providing some collisional mechanism for dissipation of the excitation energy. As a result, the average lifetime of the excited state is reduced by the same fraction as is the intensity of fluorescence. This has been verified experimentally by Bailey and Rollefson (1953), who studied the lifetime and intensity of fluorescence of fluorescein, quinine, and acridone in the presence of quenchers. Franck (1951) has discussed the implications of this concept for photosynthesis. The fluorescence yield of chlorophyll in green leaves is less than 1 % of its value in organic solvents. Thus the energy of excitation of chlorophyll must reside in the fluorescent state only 1 % as long in a leaf compared with an isolated chlorophyll molecule in dilute solution. This fact has important consequences for testing various proposals for the mechanism of energy utilization in photosynthesis.

Gaffron (1927) reported that chlorophyll sensitizes certain photo-oxidation reactions with a quantum yield of about one under conditions where chlorophyll fluorescence is not quenched by the reactants (Livingston and Ke, 1950). This must mean (Franck, 1951) that chlorophyll must transfer its energy to the oxidation-reduction system by an excited state other than the fluorescent state; for if the sensitized chemical reaction were competing with fluorescent emission for the energy in the fluorescent state, the intensity of fluorescence should be lowered when the reaction takes place. This set of facts constitutes one of the principle pieces of evidence for the existence of a metastable state of chlorophyll which is formed from the fluorescent state in less than 10^{-10} seconds.

Kautsky (1931) observed that the chlorophyll fluorescence of a green leaf undergoes very rapid changes in intensity during the first minutes of an illumination period, the so-called induction period of photosynthesis. This observation, since confirmed and extended by many workers, must mean that rapid changes occur either in the number of chlorophyll molecules or in the nature of the intermolecular environment surrounding each chlorophyll molecule. Fluorescence is one of the few types of measurement rapid enough to follow these transient effects, some of which occur in less than a tenth of a second. Since the environment may include water, protein, photosynthetic substrate, inhibitors, enzymes, or indifferent substances, the interpretation of the fluorescence anomalies is not a simple matter. Franck (1949b) has shown in an extensive review how the fluorescence measurements together with other types of observations lead to significant conclusions about the structure of the chlorophyll-protein complex in plants and about the natural mechanisms by which the photosynthetic apparatus of a plant adapts to changes in the environment.

Sensitized fluorescence has been discussed in Section I.2.*c*.(1). Mention was made there that chlorophyll *a* fluorescence in plant plastids is sensitized by auxiliary plastid pigments. This means that the energy absorbed by the other pigments is made available to chlorophyll *a* not only for fluorescence but for any other purpose for which chlorophyll uses energy in its fluorescent state. If most of the energy, say 99 % for example, placed in the fluorescent state of chlorophyll by direct absorption is transferred to the metastable state, then the occurrence of a measured amount of sensitized fluorescence of chlorophyll is also accompanied by the infiltration of 99 times that amount of energy into the metastable state. Thus fluorescence, representing just a small fraction of energy utilization, serves as a monitor for considerably more energy.

3. CHEMILUMINESCENCE

The utilization of chemical reactions for the production of energy is a familiar phenomenon. The reason that reactions take place is that the products represent a lower state of chemical potential energy than the reactants. Some of this loss in potential energy becomes available in usable forms, such as heat, the mechanical energy of expansion, or the electrical energy of batteries. In some rare cases energy is evolved by the chemical system as light, and the phenomenon is called chemiluminescence. The light emitted is always of a wave length corresponding to an electronic transition of some molecule participating in the reaction. In other words, as a result of the chemical rearrangement of the participating substances some molecule is left in an excited state, and this molecule returns to its ground state in a secondary process by radiating the energy of excitation as light of the appropriate wave length. If the excited state is the same state which the normal molecule reaches by absorbing light, then the chemiluminescence spectrum is identical with the fluorescence spectrum. Since the ability of an excited molecule to emit light is independent of the method by which the excitation occurred, chemiluminescent substances must invariably be molecules that are able to fluoresce. The converse is not true since a special set of circumstances is necessary for a fluorescent substance to be excited electronically as the result of a chemical reaction.

In general, chemiluminescence is not well understood and its occurrence cannot be predicted. The known examples have been identified mostly as the result of random observations. The subject has fascinated biologists for ages because of the striking aspects of bioluminescence (Harvey, 1952) and has challenged chemists and physicists as a problem in the mechanism of energy transfer. Another reason for interest in chemiluminescence is its possible connection with photochemistry. These two processes are the reverse of each other in their overall description. In one, light is absorbed so that a reaction may occur that otherwise would not take place. In the other, a spontaneous reaction occurs and light is evolved. There has been some speculation that the concept of reversibility might also be extended to some of the detailed steps in the two processes and that research in one might give results suggestive for the other. A striking example is the chemiluminescence of chlorophyll that can be observed up to several minutes following a period of photosynthesis by green plants (Strehler and Arnold, 1951). This phenomenon is believed to be connected with some back-reaction of freshly formed photosynthetic products, and a detailed study of the chemiluminescence should have some important bearing on the nature of the linkage of chlorophyll with its substrates in photosynthesis.

One necessary condition for a chemiluminescent reaction is that it be strongly exothermic. The energy of the reaction must at least equal the energy for electronic excitations, which in the visible region is greater than 40 kilocalories per mole. It is not surprising, therefore, that most of the known chemiluminescent reactions are such rich energy producers as oxidations and peroxide decompositions. Some examples are the decomposition of hydrogen peroxide in the presence of riboflavin (Strehler and Shoup, 1953); the decomposition of tetralin hydroperoxide in the presence of chlorophyll or other porphyrins (Rothemund, 1938; Linschitz and Abrahamson, 1953); and the reaction of oxygen with the phosphorescent state of acriflavine and other fluorescent dyes (Kautsky and Müller, 1947).

Several interesting analytical applications have been made of chemiluminescence. Luminescent bacteria have been used since 1889 for the detection of minute amounts of oxygen (Beijerinck, quoted by Harvey, 1940). Adenosine triphosphate has been found to be necessary for the luminescence of fireflies, and a quantitative assay for this compound, based on the intensity of luminescence of a firefly enzyme extract, has been developed by Strehler and Totter (1952).

III. Special Experimental Techniques

Various pieces of equipment are necessary for a complete photochemical investigation. There must be a light source, a set of lenses for rendering the light parallel or for focusing the light on the reaction cell, screens or a light-tight box for housing the sample to keep out stray light from the room, filters for isolating particular wave lengths, the sample holder, a light-sensitive detector properly shielded from stray light, and perhaps electrical equipment for amplifying or recording the output of the detector. It is useful to mount as many of the components in the optical path as possible on a single frame. Optical benches, which are heavy steel channels on which optical parts can be mounted and placed at continuously variable distances from each other, are helpful for achieving compact mountings. The particular design of the apparatus must of course be made in each case with regard to the type of sample. If the reaction is to be analyzed continuously during the illumination, the analytical method will impose the chief limitations on the optical geometry. The reaction cell also must be chosen with reference to the type of sample. If possible it is desirable to use a cell with plane parallel windows. Such a cell used with a well collimated beam simplifies many of the optical measurements. The cell windows, and also the optical lenses, must be of a material which transmits the wave lengths under investigation. Optical glass cuts off ultraviolet light below about 3500 A, pyrex below 3200 A, and fused silica below 2000 A. A more detailed discussion of some of the experimental aspects is given below.

1. LIGHT SOURCES

Practically every type of light source has been used for photochemical investigations. The emphasis has often been placed on high intensity lamps so that a plentiful supply of quanta is available to produce a measurable amount of reaction. A hypothetical 1000-watt lamp that converted 100 % of its input energy into 5000 A light would yield only one-fourth of an einstein per minute. The amount of available light energy for photochemistry must be scaled down from this value (1) because no lamp is 100 % efficient in conversion to radiant energy, (2) because every lamp has its radiant light distributed over different wave lengths not all of which can be used photochemically, (3) because in a practical experiment only a fraction of the light from a lamp can be focused in the direction of the reaction cell even with the use of lenses and reflectors, (4) because much of the useful light is lost by absorption in any filtering system introduced to monochromatize the light, and (5) because the reaction sample will in general not absorb 100 % of the radiation falling on it even at the optimum wave length. As a result of all these limitations, it is unusual to expect the absorption of more than 10^{-4} einsteins per minute of actinic light. Another important aspect of a light source is its geometry. A compact source is easier to collimate than is a diffuse source. The modern experimenter is fortunate in having available a large variety of commercial lamps covering a wide range of characteristics such as intensity, spectral distribution, size and shape, and operating power supply. The lamp manufacturing companies should be consulted for the detailed statements of characteristics of their products.

a. Continuous Spectra

Light sources are called continuous if the output is a smooth function of wave length. The principal type of continuous light source is a heated body, such as in a tungsten filament lamp or a carbon arc. Projector lamps and automobile headlight lamps have been frequently used because of the concentration of the light in a small section of the filament.

Continuous sources are most useful where wave length discrimination is not important. They are convenient even in other cases because they are inexpensive and can be operated without special power supplies. High stability can be achieved with low voltage lamps by running them from storage batteries. If used in conjunction with a filtering system, a single lamp can provide light at any desired wave length within its range. Since most filament lamps have glass envelopes, they do not transmit much ultraviolet. The cut-off point is from 3200 A to 3500 A, depending on the composition of the glass. Filament lamps are energetically inefficient because most of their output is in the unusable infrared.

Hydrogen discharge lamps are often used as continuous sources in the range from 2000 A to 4000 A.

b. Line Spectra

Line sources are lamps which produce most of their radiant energy at discrete wave lengths. Such a source, when viewed through a spectroscope, has the appearance of a series of more or less narrow lines or bands. These lamps are devices for exciting the emission spectra of one or more gaseous or volatile elements, and the spectral distribution of the lamp is a property of the excitable element. The principal advantages of line sources are (1) the ease of obtaining monochromatic light and (2) the higher light intensities per unit of power input.

For photochemical work the most common type of line source is the mercury lamp. Low pressure mercury lamps produce most of their radiant output at 2537 A. Of course these lamps have quartz or fused silica envelopes to transmit this radiation. These lamps are useful for bactericidal purposes and for experiments on protein inactivation because of the high absorption of 2537 A by proteins. The medium pressure mercury lamps are used for illumination in the near ultraviolet and in the visible. The principle wave lengths produced are from the group 2537, 3126, 3650, 4047–4078, 4358, 5461, and 5770–5790 A. These lamps, including the popular H1 and H4 series of several American manufacturers, come with either glass or quartz envelopes, only the latter transmitting the deeper ultraviolet lines. Cadmium or cadmium-mercury lamps have often been used to extend the coverage of the visible region toward the red; they have a prominent line at 6439 A. Sodium lamps and hydrogen lamps have also been used for line irradiation in the visible.

Most of these atomic vapor lamps must be operated with a specially designed power supply. Manufacturers' specifications should be consulted for details of operating characteristics including spectral distribution.

c. Flash Illumination

Single intense bursts of light have been used for photochemical studies only recently, although the equipment has long been in common usage among photographers. Magnesium flashbulbs may be used, but they are very irreproducible in both total flash intensity and in flash duration. The average length of these flashes is of the order of a hundredth of a second. A better regulated flash can be obtained by discharging a condenser through a tube filled with one of the rare gases. The total intensity of the flash depends on the voltage and capacity of the condenser; and the mean duration, depending on the combined electrical characteristics of the condenser and the discharge tube, may be made as low as several microseconds. The reader should consult the original literature for details of the electronic circuitry (Porter, 1950; Marshall and Davidson, 1953; Livingston and Ryan, 1953).

2. Filters

The total irradiation of a lamp may be used for preliminary photochemical experiments; more systematic investigations often require filtered light. There may be some interest, as we have seen above, in studying the dependence of the quantum yield on wave length. In fact, quantum yield determinations can be made only with more or less monochromatic light because the measured energy of the incident radiation can be converted to the number of incident quanta by Eq. (1) only if the frequency has a definite value. An even more significant reason for working with light of controlled wave length is that the character of the reaction may change with wave length. The formation of vitamin D_2 by the ultraviolet irradiation of ergosterol is a complicated sequence of photochemical steps involving the formation of at least two photolabile intermediates (Setz, 1933). The ergosterol, the intermediates, and the product vitamin D_2 are all unstable to light, and each of these substances has its own action spectrum. A feasible scheme for producing the vitamin requires a careful selection of the irradiating wave length so that the product will not be decomposed by the light used to form it. The intermediates lumisterol and tachysterol were isolated by irradiating ergosterol at wave lengths at which the intermediates themselves absorb relatively weakly.

Some baffling aspects of the photoperiodic behavior of plants were clarified with the discovery by Borthwick *et al.* (1952, a and b) that two different pigments, photochemically interconvertible, are involved in such control functions as seed germination and flowering. Germination of lettuce seeds is promoted when one pigment absorbs, the absorption maximum being at 6600 A. Germination is inhibited when the other pigment absorbs, the absorption maximum being at 7300 A. Light at any intermediate wave length sets up an equilibrium distribution of the two pigments dependent on the relative absorptions.

Another mystery that has recently been solved with the help of monochromatic irradiation has centered about the reversibility of the photobleaching of the visual pigment rhodopsin. Chase and Smith (1939) had observed that solutions of rhodopsin bleached in yellow light would not regenerate the pigment in the dark, whereas solutions bleached with yellow and blue light showed regeneration in the dark. Hubbard and Wald (1952) have now recognized that the end products of the irradiation of rhodopsin solutions with yellow light are opsin and all-*trans* retinene. The resynthesis of the pigment in the dark requires the combination of opsin with a *cis*-isomer of retinene called neoretinene *b*. The all-*trans* isomer can be converted to the *cis* by a second photochemical reaction which requires blue and near ultraviolet light absorbed by the retinene.

The two general methods for isolating a given spectral region from a polychromatic source are (1) resolution of the light into its spectral components and (2) selective absorption of the unwanted components. The first method takes advantage of the light dispersing properties of prisms or gratings, and it constitutes the principle of the monochromator and the spectroscope. One of the earliest observations of bacterial photosynthesis was made by Engelmann in 1888 (quoted by Rabinowitch, 1945, p. 99) by shining a spectrum upon a culture of the bacteria. The culture grew only in those wave lengths absorbed by bacteriochlorophyll. Here was an entire action spectrum determined in one experiment with the help of a small spectroscope. Recent applications of monochromators have been reported by French *et al.* (1947) for photosynthesis studies and by Borthwick *et al.* (1952a) for investigations of photoperiodic phenomena in plants. Although monochromators are now available commercially from several manufacturers of optical equipment, they have not been used frequently in photobiology because of their cost and because of the sacrifice in light intensity which their use generally requires. These instrument, however, are unsurpassed in the continuously variable control they offer for wave length selection and wave length band thickness.

The use of filters for selective absorption of the undesired spectral components is much more common. The fine resolution of spectra so commonly demanded in spectroscopy is rarely necessary in photochemistry. The common electronic absorption bands in condensed systems are so broad that inexpensive filters can usually be used for isolating a band narrow enough to avoid an appreciable change in the absorption coefficient of any one molecule and to avoid overlap with the absorption regions of other components. Especially if a mercury lamp is used the principal lines are sufficiently separated so that a filter can easily be selected to transmit only one of the lines or a particular group of adjacent lines.

For many years the largest variety of filters available in America were the colored glass filters (Corning) and the Wratten gelatin filters (Eastman Kodak). The Zeiss and Schott filters are among the principal European lines.

The manufacturers provide on request the detailed transmission curves for their filters. These filters are particularly useful for cutting out radiation below or above prescribed wave lengths. When used in pairs they can be used to transmit light in narrower bands, but each additional filter means a sacrifice in transmitted intensity, even for the desired wave length. In particular, combinations have been worked out for isolating the various mercury lines. Heat resistant filters which absorb infrared radiation are especially useful in preventing the heating of a sample irradiated with a filament lamp. Many of the glass and gelatin filters have a small fluorescence, which might interfere in low level fluorescence and phosphorescence detection.

Since the war a new type of filter has become available which has as its essential component one or more layers of thinly deposited metal film protected by a sandwich of transparent glass. These filters, known as interference filters, transmit only very narrow bands the wave length of which depends on the thickness and index of refraction of the deposited film. They are available from a number of manufacturers (such as Baird, Bausch and Lomb, Farrand) over the entire range from the near ultraviolet to the near infrared. They are distinguished by their narrow band of transmission, in the order of 50 A in some cases, and by their very high transmission at the peak of the band, as high as 80%. In both respects they surpass the older types. These interference filters are excellent for use with either continuous light or with line sources. For the latter purpose the filters are matched by the manufacturers to the important mercury, sodium, or hydrogen lines. Since these filters operate on the interference principle they transmit not a single band, but all integral sub-multiples of a fundamental wave length. A conventional filter may sometimes be used in conjunction with the interference filter to remove the undesired sub-multiples. An interference filter should be used fairly normal to the light beam because the wave length of the peak transmission changes at more oblique angles.

A solution filter is another type frequently used, but such a device is not very convenient. Solutions of acetic acid are commonly used to absorb all ultraviolet radiation below 2537 A, and copper sulfate solutions are commonly used for absorbing the infrared above 7500 A. Various recipes for filter solutions which transmit specified wave length regions are given by Bowen (1946) and by Noyes and Leighton (1941).

Neutral density filters are often convenient for reducing the intensity of all wave lengths by a constant factor. Such filters can be purchased from optical and photographic supply houses or can be made in the laboratory. Various types of neutral density filters are suspensions or films of colloidal carbon, uniformly exposed and developed photographic plates, partially crossed Polaroids, and wire mesh screens painted black to eliminate reflections. Any of these filters should be tested for absence of wave length discrimination by measuring their absorption at different wave lengths in a photometer.

3. Detectors

The non-chemical types of detector measure intensities which can be converted by calibration to ergs per second. It is thus important to have a constant intensity light source and to know the exact duration of an illumination in order to calculate the total integrated energy. The number of ergs can be converted to einsteins by Eq. (19),

$$\text{number of einsteins} = 8.35 \times 10^{-17} \times \lambda \times (\text{number of ergs}) \qquad (19)$$

where λ is the wave length in A.

a. Non-selective Dectors

The standards for radiation detection are those which respond to the total energy of the radiation, independent of wave length. The most common non-selective detector is the thermopile. This consists of a series of junctions of two dissimilar metals. Alternating junctions are soldered to a thin metal foil which is later blackened with lampblack to absorb all incident radiation. The thermoelectric potential between these hot junctions and the junctions not exposed to the radiation can be measured with a sensitive galvanometer, the deflection of which is proportional to the intensity of incident radiation. Thermopiles can be purchased from the leading electrical instrument houses. References for construction of thermopiles have been given by Daniels (1936).

A thermopile is usually calibrated by measuring its response to the light from a standard lamp. Such a lamp can be obtained from the National Bureau of Standards together with a certificate describing the conditions for calibration. The thermopile galvanometer readings can thus be converted to ergs per second. One of the precautions necessary in the use of a thermopile is to filter out the infrared from a photochemical light source, particularly if white light is used, because most of the total energy of a filament lamp to which a thermopile responds is in the chemically inactive infrared. The calibration with a standard lamp, however, is performed with the total unfiltered light of the lamp.

The blackened surface of a thermopile is protected from convection currents with a transparent window of quartz, fluorite, or glass. Precision measurements must allow for the difference in the reflective and absorptive losses at this window arising from the difference in wave length distribution of the standard lamp and the photochemical light. Standard books on photochemistry should be consulted for the details of these corrections (Bowen, 1946; Noyes and Leighton, 1941).

b. Selective Detectors

(*1*) *Photoelectric detectors.* It is generally more convenient to use a photoelectric detector such as a photronic cell, a photoelectric cell, or a photomultiplier tube. Photomultiplier tubes can often be used without an electronic amplifier because of their internal amplification. The sensitivities of these devices depend on wave length. The manufacturer's specifications should be studied in selecting a suitable cell for each purpose. For some experiments it is sufficient to know that the response of the detector is proportional to the light intensity. But if measurements are to be made at different wave lengths, as in the determination of an action spectrum, the detector should be calibrated against a thermopile by comparing the response of each to the same filtered radiation at each wave length.

An experimental method can be used to compensate for the wave length dependence of the sensitivity of detectors. This method, known as heterochromatic photometry, has been described by a number of authors (Harrison and Leighton, 1931; Bowen, 1936; Pringsheim and Vogel, 1943). Advantage is taken of the fact that the fluorescent yield of many substances is independent of the wave length of excitation. Such substances can be used as "quantum converters." A fixed amount of fluorescence will be emitted for each quantum of light absorbed by the substance. In practice, the fluorescent substance is prepared in a thin film concentrated enough to absorb all the radiation incident upon it but dilute enough so as not to absorb any of its own fluorescence. The film is mounted so that its front faces the incoming beam and its rear faces the photocell. The photocell always "sees" the same wave length, that of the fluorescent light, regardless of the wave length of the incident beam. The intensity of the fluorescent light reaching the photocell, under ideal conditions, is proportional to the number of quanta incident upon the film per second. Some quantum converters that have been described in the given references are: (1) a mat of uranyl sulfate crystals suspended in paraffin, suitable for incident radiation from 2500 A to 3200 A; (2) a shallow aqueous solution of the dye esculin, suitable for the range from 2500 to 3660 A; and (3) a film of rhodamine B dissolved in glyptal varnish, suitable from the far ultraviolet to 5900 A.

(*2*) *Chemical actinometers.* There are some photochemical reactions whose quantum yields are so well known that an analysis of the extent of the reaction gives an immediate measure of the integrated amount of energy falling on the reaction cell. A device in which such a reaction is carried out is called a chemical actinometer. Actinometers are not primary detectors, but they can be calibrated against a thermopile or in some cases by simple reference to the literature. The advantages of actinometers are (1) that no electrical measurements need be made; (2) that a vessel of any size or shape could be adapted to hold the actinometer reactants; and (3) that occasionally an actinometer reaction can be found which has the same action spectrum as the reaction under investigation. For best results the actinometer solution should be concentrated enough to absorb practically all the radiation incident upon it. If the light source is sufficiently constant, the light intensity can be measured either before or after the main reaction is carried out by placing the actinometric solution in the reaction cell. This procedure simplifies many geometrical problems, but it is not the only way in which actinometers may be used.

The most widely used actinometric reaction is the decomposition of oxalic acid photosensitized by uranyl ion. Leighton and Forbes (1930) have measured the quantum yield of this reaction over the range from 2540 A to 4350 A and found that it has almost a constant value of about .60 over most of this range with a dip to .49 at 3600 A. The temperature coefficient is about .3 % per degree. Their recommended starting solution is .01 molar uranyl sulfate and .05 molar oxalic acid. These concentrations may be lowered at the lower wave lengths, where the absorption coefficient is very high. The solution should be well stirred during the exposure. The extent of reaction can be measured after a timed exposure by titrating the residual oxalic acid with permanganate in acid solution. A modification of the original procedure has been described by Bowen (1946).

Harris *et al.* (1935) studied the photochemical conversion of malachite green leucocyanide to malachite green as an actinometer reaction. The work has been confirmed and extended by Calvert and Rechen (1952). The reaction takes place with a quantum yield 1.00 over the temperature range from 7 to 27° and over the wave length range from 2480 A to 3300 A. The reaction is analyzed very conveniently by measuring the absorption of the product malachite green in a photometer at 6200 A. The method applies to intensities below 10^{14} quanta per square centimeter per second.

Gaffron (1927) discovered that chlorophyll sensitizes the oxidation of allylthiourea with a quantum yield of 1.0. Warburg and Schocken (1949) modified the reaction to make it suitable as an actinometer. 200 mg of thiourea are dissolved in 5 cc of pyridine, to which is added either 2 mg of ethyl chlorophyllide or 10 mg of protoporphyrin. The chlorophyllide is an efficient absorber and sensitizer in the red, yellow, and blue; and the protoporphyrin is effective in the green or blue. Since the reaction is followed manometrically by observing the disappearance of oxygen, a Warburg manometer vessel may be used. At an intensity of about 3×10^{14} quanta per second the quantum yield is reported to be $0.98 \pm .05$ from 6600 A to 5050 A. This actinometer, with ethyl chlorophyllide, is very suitable in connection with studies of chlorophyll-sensitized reactions because the actinometer fluid absorbs the same wave lengths as the reactant mixture under study.

The chlorophyll-sensitized oxidation of phenylhydrazine by methyl red

(Livingston and Pariser, 1948) has been used as an actinometer reaction. Under proper conditions the quantum yield approaches 0.50 at wave lengths absorbed only by the chlorophyll. The extent of reaction can be followed spectrophotometrically.

4. Fluorimeters

A fluorimeter is an instrument that provides a light source, a sample holder to receive incident radiation, and a detector to measure the intensity of fluorescent light. Most measurements are relative and are compared with some standard substance by either a double beam technique or by successive observations with a single beam. Such an instrument can be readily assembled from the component parts, or it can be purchased as a unit. Many colorimeters and photometers can be adapted for fluorescence measurements. The various devices used in photometry for measuring relative intensities can be used for fluorescence as well. Since fluorescent light is emitted in all directions, the detector may be placed at any convenient angle with respect to the incident beam. The detector should be provided with a filter which absorbs the wave length of the light source and transmits the fluorescent light. If a sample is irradiated at the peak of its first visible absorption band there may be some difficulty in finding a filter which will pass the fluorescent light but absorb the incident light. It is therefore advisable, where possible, to irradiate in the second absorption band (shorter wave length) or on the short wave length shoulder of the first band. This principle is used in visual fluorescence detection, where ultraviolet light is used for excitation. The filter should of course cut out all wave lengths of the irradiating beam and, for work with concentrated solutions, should cut out all wave lengths that could be absorbed by the sample. This second precaution avoids problems of reabsorption of the fluorescent light.

If a fluorescence spectrum is to be measured, the detector can be provided in turn with different narrow pass filters to cover the entire range of the spectrum, or the detector can be replaced by the entrance slit of a monochromator or spectrograph.

An excellent survey of problems of measurement of both fluorescence and phosphorescence is given in the monograph by Pringsheim (1949).

5. Rigid Medium Techniques

It is sometimes possible to detect unstable primary photochemical products by working under conditions where the normally ensuing dark reactions cannot take place. One such condition is low temperature. The photochemical step itself is not influenced by temperature, but most non-photochemical reactions occur more slowly at lower temperatures. Another favorable condition is a viscous or rigid medium. Dark reactions that require the diffusion of two molecules toward each other can be slowed down or stopped completely in a viscous solution, in a glassy solvent, or in an adsorbed film. Sometimes a combination of these two conditions has been used for identifying intermediates and thus elucidating important features of the reaction mechanisms.

Broda and Goodeve (1941) studied the bleaching of the visual pigment rhodopsin in a glycerol-water mixture which vitrified as a glass at dry ice temperatures. They found that the product of irradiation of this glass at low temperatures was an intermediate substance, since named lumirhodopsin, stable at low temperatures but unstable at room temperature with respect to conversion in the dark into retinene and opsin, the first observed products of irradiation of rhodopsin solutions at room temperature. Wald *et al.* (1950) studied this same reaction at room temperature with a dried film of rhodopsin adsorbed on gelatine. They also found lumirhodopsin as the only direct photoproduct, but the lumirhodopsin was converted in the dark into a second intermediate, metarhodopsin, in the course of minutes. The metarhodopsin was stable indefinitely on the dry film, but reacted quickly to give retinene and opsin in the dark when water was added to the film.

Linschitz and Rennert (1952) were able to form a reversibly bleached form of chlorophyll by irradiating chlorophyll at liquid nitrogen temperature in a glassy solvent made up of ether, isopentane, and absolute ethanol. An even more striking case reported by these same authors was the formation of an intermediate reaction product between chlorophyll and quinone when their solution in the same glassy solvent was irradiated at low temperatures. The intermediate product, interpreted by the authors as a mixture of the two free radicals semi-quinone and monodehydrochlorophyll, was stable indefinitely in the dark as long as the solvent was kept glassy. Lewis and Lipkin (1942) studied a number of free radicals and ionized organic molecules formed by irradiating organic dyes in rigid media. They worked out recipes for preparing glasses at various temperatures. At liquid nitrogen temperature they used mixtures of isopentane and methyl cyclohexane or mixtures of ether, isopentane, and alcohol. At dry ice temperatures they prepared glasses from glycerol, triethanolamine, sulfuric acid, or phosphoric acid. Room temperature glasses were made from boric acid or glucose. Phosphorescence of organic dyes is observed if the dye is either dissolved in a glass or adsorbed on a surface like silica gel or alumina.

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Chapter 2

LIGHT SCATTERING

Gerald Oster

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I. Introduction

The biological literature is replete with qualitative descriptions of light scattering phenomena. Thus, one reads reference to a solution being opalescent, turbid, milky, cloudy, or exhibiting a Tyndall beam. The appearance of the solution may change depending on the lighting conditions under which the observations are being made. All these qualities may be characterized quantitatively. The theory of light scattering by large molecules in solution has advanced sufficiently far that given good experimental data it is usually possible to characterize the size, shape, and interactions of biological molecules in solution. (The light scattering method has an added feature in that the solution remains undisturbed during the observations and changes with time can be easily followed out.)

Although Putzeys and Brosteux determined the relative molecular weights of several proteins in solution as early as 1935, it is only within the past ten years that many of the potentialities of the light scattering method have been exploited. The main impetus came from Debye (1944, 1947) who first applied the method to solutions of synthetic high polymers. Many papers on the subject have appeared during the past ten years but very few contain certain details which are necessary for the successful carrying out of light scattering measurements. In this chapter the theoretical basis for light scattering is outlined particularly as it applies directly to the experimental techniques and the interpretation of data. Illustrations are taken mainly from systems of biological importance. The difficulties

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of the light scattering methods described in this chapter are those personally encountered by the author and may be well known to other workers in the field but are seldom discussed in sufficient detail in specialized papers on the subject.

II. Theory of Light Scattering and Its Applications

1. Introduction to the Theory

Leonardo da Vinci (Ca. 1500) was the first to appreciate that the blue of the sky (when observed in a direction away from the sun) is due to light scattering. It was necessary, however, to wait until the advent of the theory of electromagnetic radiation in the late nineteenth century before a quantitative theory could be formulated. The essential idea of the nature of light scattering is contained in the work of Rayleigh (1871, 1881). Associated with light is a periodically varying electric field. When the light falls upon matter the field induces a polarizability which oscillates with the same frequency as the incident light. The material then acts as a secondary source of light and hence the incident light is scattered in various directions. The cloud of electrons in the matter which had been set into oscillation by the incident light now behaves in a similar manner to a radio antenna and will send off an amount of electromagnetic energy, the intensity, determined by the size of the oscillators and their number and by the frequency of their oscillations (i.e., by the wave length of the incident light).

Subsequent developments in the theory were concerned primarily with the interactions between the scattered wavelets from non-independent scattering particles, as in non-ideal solutions, or from the same particles where the particles are not small compared with the wave length of the incident light.

Comprehensive reviews of the theory of light scattering have appeared in recent years (Oster, 1948, 1949, 1950a; Doty and Steiner, 1950; Tonnelat, 1950; Doty and Edsall, 1951; Riley and Oster, 1951). A complete understanding of the derivation of light scattering formulae requires an elementary knowledge of electromagnetic theory and of the thermodynamics of solution. Lack of such knowledge, however, does not preclude one from carrying out significant experiments in light scattering as long as the limitations of the formulae to be applied to the data are sufficiently appreciated.

2. Small Independent Particles

When a beam of light traverses a light-scattering system its intensity is decreased by virtue of the energy withdrawn from the beam in the form of scattered radiation. For colorless particles the logarithm of the fractional decrease in the transmitted intensity I is given by the turbidity τ where $I = I_0 \exp(-\tau l)$, I_0 being the initial intensity and l the path length of the scattering system. In terms of logarithms

$$\tau = \frac{2.303}{l} \log_{10} \frac{I_0}{I} \tag{1}$$

and τ is in reciprocal centimeters if l is given in centimeters.

For independent particles which are optically isotropic and are sufficiently small compared with the wave length λ of the incident light, application of the Rayleigh theory gives (Oster, 1948, Sec. II A1)

$$\tau = HcM \qquad H = \frac{32\pi^3 n_0^2}{3N\lambda^4} \left(\frac{n - n_0}{c} \right)^2 \tag{2}$$

where c is the weight concentration of the particles in g per ml, M is the molecular weight, N is Avogadro's number (6.03×10^{23}), n_0 is the refractive index of the medium, and n is the refractive index of the solution. The refractive index increment $n - n_0/c$ is a constant independent of the concentration and for most biological materials in water is in the neighborhood of 0.17. Insertion of numerical values into Eq. 2 gives a value of H equal to about 3×10^{-6} when the wave length of the light used is the green line of the mercury spectrum, namely 546 mμ (5.46×10^{-5} cm).

For aqueous solutions of proteins, polysaccharides, and lipids it is permissible to regard the particles as "sufficiently small" so that Eq. 2 is applicable if the largest dimensions of the particles are less than about one-tenth the wave length of the incident light. Thus Eq. 2 is applicable to visible light scattering systems of spherical particles possessing molecular weights up to about five million. Independence of the particles is insured if the light scattering data is extrapolated to infinite dilution.

For systems exhibiting a low value for the turbidity (c and/or M are small) then $\tau l \approx (I_0 - I)/I_0$. Since the difference in intensities relative to the incident intensity can usually be determined to only a few per cent this introduces a large error in the value of τ for feebly scattering systems. In these cases the usual procedure is to measure the intensity of scattering at right angles to the incident beam and compare this intensity with that for a system of known turbidity. We shall return to this point later when considering the problem of the determination of absolute turbidities.

For a polydispersed system, that is, a solution containing particles of the same material but which are of different sizes, the molecular weight M in Eq. 2 is an average of the molecular weights of the particles. More specifically, it is the so-called weight average molecular weight given by $\sum \nu_i M_i^2 / \sum \nu_i M_i$ where ν_i is the number of particles possessing a molecular weight M_i and the summations are taken over all the particles. Since the number of particles (per unit volume) is proportional to their weight concentration c divided by their molecular weight M, Eq. 2 can also be written for a polydispersed system as

$$\tau = A \sum \nu_i M_i^2 \tag{3}$$

where A contains the numerical and optical constants of Eq. 2. Eq. 3 is useful in following the kinetics of coagulating and polymerizing systems. Thus, it has been shown (Oster, 1947) that for a system in which the particles are aggregating in a random fashion, i.e., any size particle or aggregate is combining with any other size particle or aggregate, the turbidity will increase linearly with time. Similarly, if the particles are aggregating by nucleation, i.e., aggregates are being built up about active centers, the turbidity will increase quadratically with time. In this manner, one can determine the nature of the coagulating process and, furthermore, the rate constants for the elementary aggregation steps can be calculated. The slow aggregation of serum albumin has been followed by light scattering (Bier and Nord, 1949) and the turbidity was found to be linear with time. It may be supposed, therefore, that the aggregation was of the first named type. The coagulating stage of antibody-antigen reactions is probably also of this type, at least this was found to be the case in the zone of antigen excess (*cf.* Fig. 4 of Gitlin and Edelhoch, 1951). Since Eq. 3 is derived directly from Eq. 2, its applicability is subject to the same assumptions as those of Eq. 2. Hence, when the aggregates grow to sizes which are of dimensions nearly equal to or greater than the wave length of the incident light Eq. 3 is no longer applicable and a further analysis is required as discussed below in Section II, 3. Regardless of the quantitative aspects of the theory, light scattering provides a simple means for observing the onset of a coagulation process. For example, many proteins possess a minimum solubility at their isoelectric points and will aggregate at pH values in this neighborhood. In Fig. 1 is shown the relative turbidity as a function of pH for tobacco mosaic virus (Oster, 1951). The turbidity rises rapidly in the immediate neighborhood of the isoelectric point and by a variation of this method one can determine the isoelectric point with great precision. Incidentally, the addition of salt lowers the pH where coagulation appears indicating binding of ions by the virus. Sol-gel transformations are frequently accompanied by an increase in turbidity. Thus, the transformation from fibrinogen to fibrin can be followed by this means (Ferry and Morrison, 1947; Greene, 1952) as can the sol-gel transformation of gelatin when the temperature of the sol is lowered to its gel temperature. Aggregation leading to dimerization without extensive coagulation occurs in solutions of human serum albumin in the presence of mercurials (Hughes, 1950).

Equation 3 expresses one of the most troublesome features of light scattering, namely its strong dependence on particle size. Thus, if in a system there are several small particles but only a few large particles, the main contribution to the light scattering may be given by the large particles. For example, one particle will scatter as much light as ten thousand particles of one hundredth the size of the former particle. Hence, particles of dust or other extraneous matter could make the major contribution to the turbidity. It is for this reason we shall consider methods for the clarification of the solution in some detail (Section IV).

The qualitative appearance of light scattering is contained in Eq. 2, namely that the amount of light scattered is inversely proportional to the fourth power of the wave length of the incident light. Thus a turbid solution will be more opaque to blue light than, say, to red light. For this reason a blue filter is usually employed for the determination of the relative concentration of a colloidal solution in a colorimeter. Incidentally, Eq. 2 shows that the optical density D (τ equals $2.303D$ for a 1 cm path length) is proportional to the concentration as required by Beer's law if the solution is sufficiently dilute. In order to make accurate determinations of molecular weight by light scattering it is necessary that the incident light be monochromatic. If white light is employed the turbid solution will appear yellowish on transmission since blue light has been withdrawn from the beam by light scattering. The apparent color of the solution due to light scattering must be distinguished from that due to intrinsic color of the particles. Quantitative spectrophotometry involving large molecules must necessarily account for the distortions in the transmission spectra due to light scattering. These distortions are particularly severe in the blue region of the visible spectrum and throughout the ultraviolet region of the spectrum. For example, nearly 50% of the apparent absorption of tobacco mosaic

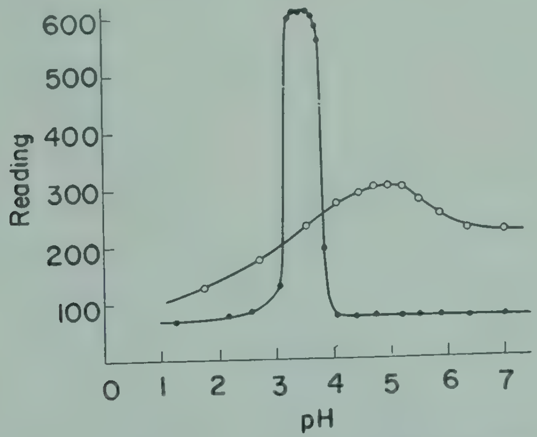

FIG. 1. The relative turbidities as a function of pH (buffered) of solutions of the ordinary strain of tobacco mosaic virus (●, virus concentration, 2.0 mg per cc) and of the PR 8 strain of influenza virus (○, virus concentration 0.005 mg per cc) (Oster, 1951).

virus at 260 mμ is due to light scattering, the remainder being due to the ribose nucleic acid which the virus possesses and which has a high intrinsic absorption at this wave length (Oster and McLaren, 1950). Even larger distortions are exhibited by influenza virus. Here the characteristic maximum at 260 mμ due to nucleic acid does not appear and it was found (Oster, unpublished) that if the virus particles were suspended in glycerol, which closely matches the refractive index of the particles so that most of the light scattering is removed, the resulting ultraviolet is that of nucleic acid alone and agrees with that calculated from the chemical analysis of the virus.

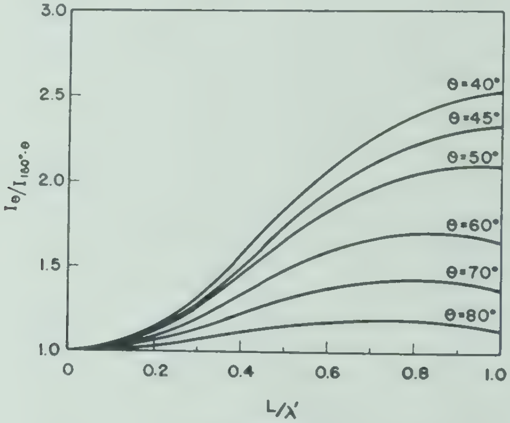

FIG. 2. Dissymmetries for thin rods.

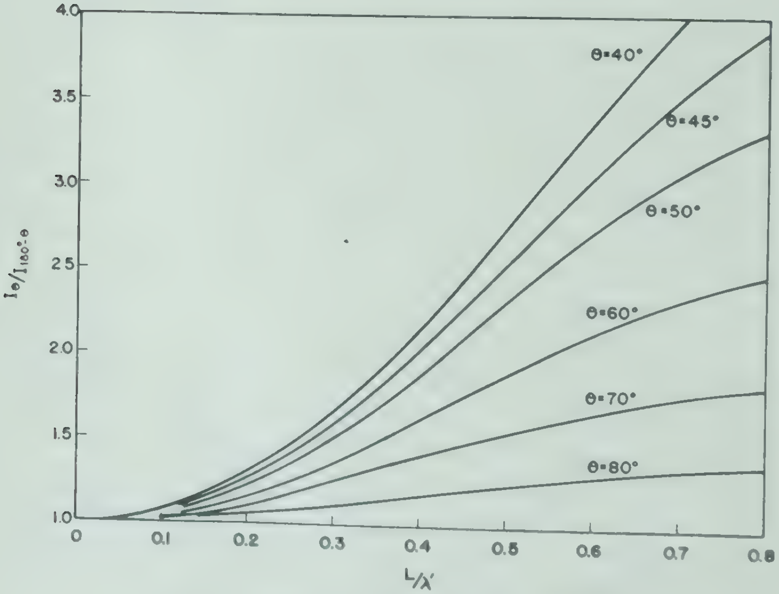

FIG. 3. Dissymmetries for random coils.

3. Large Independent Particles

For particles with a dimension of the order of the wave length of light each portion of the particle will scatter light according to the Rayleigh relation. Since the portions of the particle are in fixed positions relative to one another the scattered wavelets will interfere. We shall refer to this as intraparticle interference. The amount of destructive interference will be greater the greater the angle of observation (throughout we will take the angle of observation θ as the angle which the observer makes with the transmitted beam). That is, more light will be scattered in the forward directions than in the backward directions since there is less destructive interference in the former case than in the latter. The ratio of the intensity of light scattered at some forward angle θ to that scattered at its supplementary angle $180° - \theta$ is referred to as the dissymmetry of scattering (Debye, 1947). For a given pair of angles, the dissymmetry $I_\theta/I_{180°-\theta}$ will be greater the number of pairs of scattering elements in the particle. Thus the dissymmetry will increase the greater is the ratio of the dimension L of the particle to the wave length of the light λ'. (Note: the wave length of the light in the medium λ' is the wave length of the incident light λ divided by the refractive index of the medium.) Since a sphere of diameter L has more pairs of scattering elements than a thin rod of length L the sphere will exhibit a greater dissymmetry. The features of the dissymmetry as a function of angle, dimension, and shape are illustrated in Figs. 2, 3, and 4. In Fig. 2 L refers to the length of the rod and in Fig. 4 L refers to the diameter of the sphere. Figure 3 is that for a so-called random coil, that is, a randomly kinked polymer where the root mean square distance between the ends of the coil is given by L (see Oster, 1948, for formulae and references).

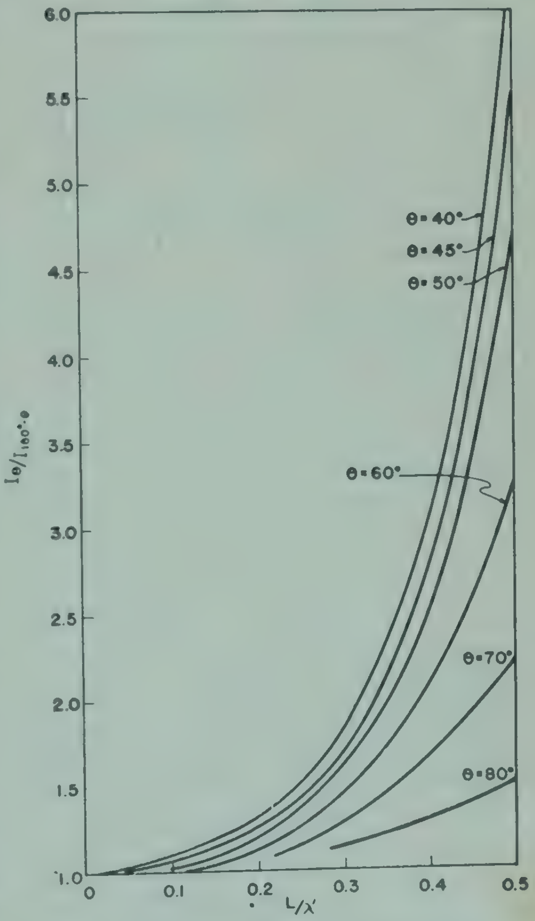

Fig. 4. Dissymmetries for spheres.

The dissymmetry for very small particles ($L/\lambda \rightarrow 0$) approaches unity. That is, the angular scattering for a Rayleigh scatterer is symmetrical. More precisely, a Rayleigh scatterer exhibits a scattering proportional to $1 + \cos^2 \theta$ but this function is symmetrical about 90°, i.e., $I_\theta/I_{180°-\theta}$ equals unity for all values of θ. As the ratio L/λ' increases, the dissymmetry minus unity becomes greater than zero and, in fact, at first varies quadratically with L/λ'. We can express the difference between the dissymmetry and unity for particles which are not too big (small L/λ') by the approximate formula

$$\frac{I_\theta}{I_{180°-\theta}} - 1 = a(L/\lambda')^2 \cos \theta \tag{4}$$

where a equals 4.38, 8.76, and 7.90 for a thin rod, a random coil, and a sphere, respectively. Values of a for other shapes are available (Riley and Oster, 1951).

When L/λ' is very large the dissymmetries approach $\cot \theta/2$ and $\cot^2 \theta/2$ for a thin rod and a random coil, respectively. Thus, suppose that the observed ratio of the intensities at 45° and 135° was 3.52, then since $\cot 45°/2 = 2.4$ we could say immediately that the particles are not thin rods. From Figs. 3 and 4 we see that the particles could be random coils of dimension $L/\lambda' = 0.70$ or spheres of dimension $L/\lambda' = 0.44$. Hence we see that a single measurement of dissymmetry at one pair of angles is not sufficient to characterize the size and shape of the particles. It is necessary to make measurements at a wide variety of angles and to see on which of the curves for the idealized shapes we obtain a constant value of L/λ' for all angles. For example, aqueous solutions of hyaluronic acid gave (for $\lambda = 546$ mμ) values of the dissymmetry of 5.65, 5.32, 4.77, 3.40, 2.39, 1.53, for θ equal to 40, 45, 50, 60, 70, and 80 degrees, respectively (Blumberg and Oster, 1954). All these results are consistent with a value of $L/\lambda' = 0.51$ for Fig. 4 but do not give a constant value of L/λ' for Fig. 3 and are obviously inconsistent with Fig. 2. Thus, we can say that the particles are spheres of diameter $L = (0.51)\ (546)/1.33 = 210$ mμ.

If the particles are ellipsoidal in form or are rods of appreciable thickness other curves must be constructed. Although formulae are available for such particles (Roess and Shull, 1947; Saito and Ikeda, 1951) there is a certain amount of ambiguity in the interpretation of the data. For example, dilute solutions of natural actomyosin exhibits a change in dissymmetry (at 45° using light of wave length 546 mμ) from its normal value of 3.4 to a value of 4.7 on the addition of adenosine triphosphate. This has been interpreted differently by two groups of authors (compare Jordan and Oster, 1948; and Blum and Morales, 1953). A further ambiguity is introduced if the particles are optically anisotropic. For example, the dissymmetry (for

$\lambda = 546$ mμ) of desoxyribose nucleic acid at 45° is 3.5 which has been interpreted as meaning that the particles are kinked rather than straight rods (Doty and Bunce, 1952). If the optical anisotropy of the particles is taken into account (Horn, Benoit and Oster, 1951), the observed dissymmetry is consistent with that expected for stiff thin rods of negative optical anisotropy. Despite the uncertainties in some special cases, however, the dissymmetry method is very useful since most molecules of biological origin are probably of simple shape and free from large optical anisotropy. In the case of virus particles where the size and shape is known from other data the dissymmetry method gives good agreement (*cf.*, Oster, 1948). Throughout this discussion we have assumed that the dissymmetry data has been extrapolated to zero concentration where interferences between particles has been eliminated.

Further complications are introduced if the particles are polydispersed. As is seen from Eq. 4 when the dissymmetries (minus unity) is small, the larger particles will make a much greater contribution than will the small ones. Since a given sample may have an unknown size distribution, the interpretation of the data becomes non-unique. There have been theoretical criteria to detect polydispersivity in a system (Benoit, 1953) but the methods have not yet been applied in practice. Extraneous particles will introduce errors especially if their contribution to the total scattering is large. Here, again, the simplest solution of the problem is to remove these extraneous particles by methods to be described.

Since the dissymmetry method characterizes only the largest dimension of the particle but not its molecular weight, in order to determine the molecular weight of the particles it is necessary to measure the turbidity. If the turbidity is sufficiently high it can be measured by transmission as described in Section IV. Frequently, the turbidity is too low to be measured by this means and the intensity of scattering as a function of angle must be measured. One procedure is to plot the observed intensities as a function of $\sin^2 \theta/2$ and extrapolate to zero angle (Zimm, 1948). The Zimm plot is a double plot in which extrapolations to infinite dilution are also made to eliminate interparticle interferences of the scattered wavelets. The justification of the Zimm angular extrapolation is contained in a more general formulation of the problem (Guinier, 1939) where the scattering due to Rayleigh scatterers must be multiplied by a power series in $\sin^2 \theta/2$ to correct for the intraparticle interferences. For small value angles we obtain for this corrective factor (Riley and Oster, 1951)

$$P(\theta) = 1 - a\left(\frac{L}{\lambda'}\right)^2 \sin^2\frac{\theta}{2} \tag{5}$$

where a is the same constant as that appearing in Eq. 4. Equation 4 was obtained from Eq. 5. It should be noted that Eq. 5 is also applicable to small angle x-ray scattering of macromolecules for which it was originally derived (*cf.* Oster, 1949, 1950a).

Due to intraparticle interference, the intensity of scattering at 90° will be less than that expected for a Rayleigh scatterer of the same molecular weight since much of the light has been scattered in the forward directions. The apparent turbidity obtained at 90° must be multiplied by the reciprocal of P (90°) to obtain the true turbidity from which the molecular weight is obtained (Eq. 1). If the dissymmetries are not too large this multiplying factor becomes

$$P^{-1}(90^\circ) = 1 + \frac{a}{2}\left(\frac{L}{\lambda'}\right)^2 \tag{6}$$

where a is the same constant as that in Eq. 3 and hence depends on the shape of the particle. Alternatively Eq. 6 can be combined with Eq. 4 to give

$$P^{-1}(90^\circ) = 1 + \frac{\sec\theta}{2}\left(\frac{I_\theta}{I_{180^\circ-\theta}} - 1\right) \tag{7}$$

Hence we see that dissymmetry measurements must be made in order to correct the apparent turbidity at 90° to give the true turbidity τ. Equation 7 implies that the multiplying factor P^{-1} (90°) is dependent only on the observed dissymmetries and does not require a knowledge of shape of the particle (contained in the constant a). This is not the case, however, since Eq. 7 is applicable only for values of the dissymmetry for which the second term on the right hand side of Eq. 7 is small compared with unity, i.e., for dissymmetries close to unity. For high values of the dissymmetry a numerical calculation is required and the shape enters into the multiplying factor. Values of P^{-1} (90°) as a function of L/λ' for various particle shapes are given in Fig. 5.

Hence we see that mere right angle ($\theta = 90^\circ$) measurements of the intensity of scattering is not sufficient to characterize the molecule if it is comparable in size to the wave length of the light. Right angle measurements of the apparent turbidity are without value if not corrected for the dissymmetry. For example, it was found that the intensity of scattering at 90° for natural actomyosin decreased upon addition of adenosine triphosphate (Mommaerts, 1950). This was interpreted to mean that the molecular weight of the particles decreased, i.e., that there was dissociation into actin and myosin. However, transmission measurements show that the molecular weight does not change under these conditions (Jordan and Oster, 1948) and this is varified by correcting the apparent turbidity for dissymmetry (Blum and Morales, 1953). As mentioned earlier, the solu-

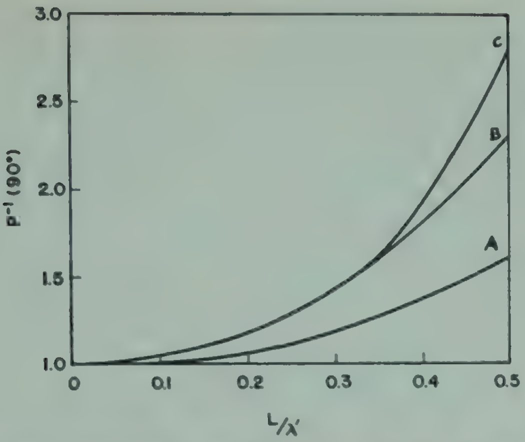

FIG. 5. Turbidity correction factors for thin rods (A), for random coils (B), and for spheres (C).

tions exhibit an enhanced dissymmetry on the addition of adenosine triphosphate due to changes in shape of the molecules and hence less light is scattered at right angles.

It is important to realize that the dissymmetry measurement gives the size and shape of the envelope of scattering material within the particle whereas the turbidity measurement gives the molecular weight of the scattering material itself. Thus, for hyaluronic acid the particles were found to be spheres of diameter 210 mμ but turbidimetric measurements corrected for the dissymmetry gave a molecular weight of 8×10^6 (Blumberg and Oster, 1954). From these two figures it can be readily calculated that the particles are swollen spheres of radius 210 mμ and contain about 400 parts of water to one part by weight of hyaluronic acid.

Although the turbidity for dilute solution of large particles can in principle be measured by transmission, the turbidity obtained does not correspond to that of Eq. 1. Due to intraparticle interferences the energy withdrawn from the incident beam will be less than that expected for Rayleigh scatterers of the same molecular weight. For very small values of L/λ' the turbidity as measured by transmission must be multiplied by a factor equal to P^{-1} (90°) given by Eq. 6 and Fig. 5 to give the equivalent Rayleigh scattering turbidity τ to be used in Eq. 1. For large particles the turbidity will no longer follow the inverse-fourth power dependence on wave length as required by the Rayleigh theory. In general, the turbidity will be proportional to $\lambda^{-4+\beta}$ where β equals zero for a Rayleigh scatterer. For very large particles (L/λ' large) β equals 1.0, 1.74, and 2.0 for rods, coils, and spheres, respectively (Oster, 1949). For intermediate values of L/λ' the values of β and of the turbidity multiplying factors must be calculated numerically and tables of such quantities exist (Doty and Steiner, 1950). The dependence of turbidity, as determined by transmission, on wave length can be used as a means of determining particle size (Heller, Klevens, and Oppenheimer, 1946; Doty and Steiner, 1950). The theoretical difficulties of intraparticle interefrence can be eliminated by making transmission measurements at sufficiently long wave lengths where β equals zero, i.e., where L/λ' is very small. For example, if the transmission measurements of dilute solutions are carried out in a spectrophotometer one makes a plot of optical density versus λ^{-4} and the density which falls in the straight line portion of the plot corresponds to the turbidity for a Rayleigh scatterer and hence the molecular weight may be determined from Eq. 1 (Oster, 1946).

For particles of dimensions close to or greater than the wave length of the incident light and resultant scattering can be calculated by means of classical physical optics. If the particles are uniform in at least one of its dimensions (e.g., the diameter of rod shaped particles such as *E. coli*) and if this dimension is close to or greater than λ' then diffraction effects will arise. These effects are seen most distinctly if the particles are confined to a surface. For example, if a parallel beam is made to pass through the large face of a petri dish in which a culture of streptococcus is dispersed on the surface of the agar, the transmitted beam will be surrounded by a colored halo. The sine of the angle θ which any color makes with the transmitted beam is inversely proportional to the dimensions of the particles. By this means the author (unpublished) has been able to quickly distinguish between capsulated and noncapsulated bacteria and to estimate the thickness of the capsule. Experiments of this general type constitute the earliest experiments on the diffraction of light and have been developed in recent years for particular application to the study of erythrocytes (for review, see Ponder, 1944). The method has been examined in detail for spherical particles of known size (Alfrey, Bradford, Vanderhoff, and Oster, 1954) and it was found, experimentally, that the diffraction is a maximum at the angle $\theta_{\max}$ where $\sin \theta_{\max} = 1.14\ \lambda/L$, L being the diameter of the spheres. Thus, for large values of L/λ the diffraction will be close to the main beam. For example, uniform particles one micron in diameter will exhibit with a mercury light source a blue ($\lambda = 436$ mμ) maximum at $\theta = 29.8°$ and a green ($\lambda = 546$ mμ) maximum at $\theta = 38.3°$. It should be emphasized, however, that the diffraction method is of use only for systems of highly uniform particle size. For a polydispersed system the diffraction maxima are very broad.

4. Non-independent Particles

In solutions which are not ideal in the thermodynamic sense, the spacial dispositions of the particles are not independent. The total intensity of scattered light will no longer be the mere summation of the intensities from the individual particles. Some destructive interference will occur and result in a decrease in intensity of scattered light from that expected for the same concentration of independent particles. One could, in principle, calculate the phase relations between the scattered wavelets of the individual particles but we can circumvent the mathematical difficulties by treating the problem as a fluctuation phenomenon. That is, in any small volume of the solution the time average of the concentration is equal to the macroscopic value of the concentration but the time average of the square of the difference between the local concentration and the macroscopic concentration will differ from zero. This reasoning leads to the Smoluchowski-Einstein equation (for derivation, see Oster, 1948, Section III, 2) for small, non-independent particles

$$\tau = H \frac{RTc}{\frac{\delta P}{\delta c}} \tag{8}$$

where R is the gas constant and $\delta P/\delta c$ is the change in osmotic pressure P with concentration. For an ideal solution, the osmotic pressure is given by the van't Hoff expression $P = RT \frac{c}{M}$ and insertion into Eq. 8 gives Eq. 2, the turbidity for a system of independent scatterers. For any solution of weight concentration c the osmotic pressure may be written in the form of a power series in c

$$P = RT\left(\frac{c}{M} + Bc^2 + \cdots\right) \tag{9}$$

where the first term on the right is the van't Hoff expression, the second term contains the so-called virial coefficient B, and the terms involving higher powers of c are multiplied by the higher virial coefficients. Insertion of this equation into Eq. 8 and rearranging, gives

$$H\frac{c}{\tau} = \frac{1}{M} + 2Bc + \cdots \tag{10}$$

At first sight it might appear surprising that light scattering gives information about the osmotic pressure of solutions. It should be recalled, however, that the method of determining osmotic pressures by means of a semipermeable membrane is a device used to determine the difference between the thermodynamic activity of the solvent in the solution with that of pure solvent. Donnan equilibrium effects arise because of the presence of the membrane whereas in other thermodynamic measurements of the osmotic pressure no such effects are present. Light scattering measurements determine the slope of the curve of osmotic pressure *versus* concen-

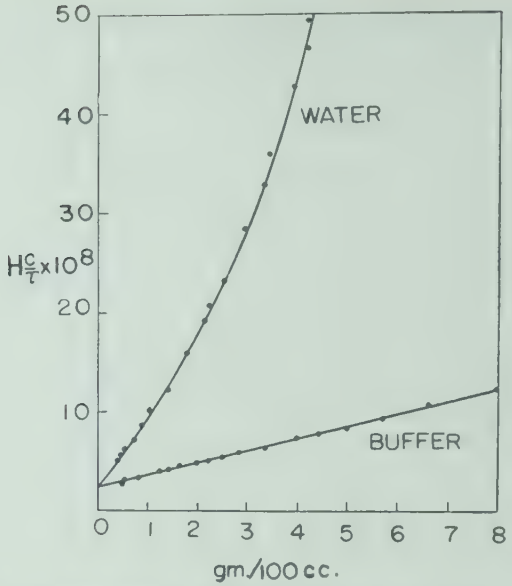

FIG. 6. Reciprocal of the specific turbidity with green light as a function of virus concentration in water and in 0.1 molar phosphate buffer (Oster, 1950b).

tration, namely $\delta P/\delta c$. In Fig. 6 is shown a plot of $H\frac{c}{\tau}$ *versus* c for tobacco mosaic virus in water and in 0.1 M phosphate buffer at pH 7.0. At infinite dilution (c approaching zero) both curves approach the same value which, from Eq. 10, is the reciprocal of the molecular weight of the virus. The linear portion of the curves have, according to Eq. 10, a slope $2B$. From Fig. 6 B for the virus in pure water is five times that for the virus in buffer. B is a measure of the interactions of the particles and of their shape. In general, B is greater the greater the repulsion between the particles and the more elongated are their shape. For example, Fig. 6 shows that salt decreases the repulsion between the virus particles (salt decreases the size of the repelling ionic double layer surrounding the particles) yet the molecular weight is unchanged since both curves have the same intercept on the ordinate. A detailed discussion of the significance of B for large electrically charged macromolecules is available (Oster, 1950b). B will decrease in magnitude in the neighborhood of the isoelectric point where repulsions between the particles are a minimum. If specific ion binding takes place the value of B will also be altered. In this way one can study rather subtle changes in interaction of proteins with other ionic species (Edsall, Edelhoch, Lontie, and Morrison, 1950).

The scattering from non-ideal solutions of large particles is quite complicated since now there are both intra- and intermolecular scattering contributions. The theory for interacting large spherical particles has been developed (Oster and Riley, 1952). Regardless of the shape of the particles, however, the dissymmetry for very dilute solutions will increase linearly with decreasing concentration and approach that expected for independent large particles as discussed earlier, and the slope of this line is measure of *B* (Oster, 1949).

III. Light Scattering Apparatus

A photometer for the quantitative measurement of light scattering contains the following essential elements: 1) a parallel beam of monochromatic light, 2) a sensitive light detector, and 3) a sample holder. Two well-designed instruments, the Phoenix (Brice, Halwar, and Speiser, 1950) and the Aminco (Oster, 1953), are commercially available. The discussion will be confined mainly to the latter instrument with which the author has had more intimate experience and which has been designed particularly for photometric measurements in biochemistry.

The light source is an AH4 125 watt medium pressure mercury lamp. This lamp is provided with a ballast which allows for alterations in current for the starting of the lamp and, furthermore, stabilizes somewhat the light output of the lamp. The lamp reaches its full brilliance after about ten minutes of operation. This lamp is particularly rich in the following spectral lines: 365 mμ (near ultraviolet), 405 mμ (violet), 436 mμ (blue), 546 mμ (green), and 578 mμ (yellow). The first-named line (or, rather, group of lines) is not normally used for light scattering but is very useful for fluorimetry. These lines are isolated by interposing the appropriate colored glass filters (Corning glass). Because of their relatively high intensities, the blue and the green lines are usually chosen for most light scattering studies. The light beam may be rendered parallel by a system of slits. Although this results in a considerable loss in intensity, the light detector, described below, is sufficiently sensitive to measure the intensity of the scattered light.

The problem of the detection of light of low intensities has been simplified in recent years by the development of the photomultiplier tube. This is a compact photoemissive cell which contains surfaces which amplify the photocurrent by secondary emission. The RCA 931-A photomultiplier tube which has its maximum sensitivity in the blue region of the spectrum produces of the order of one ampere of current when one lumen of light falls on the photoelement of the tube. Since currents as low as 10^{-5} microamps can be measured then intensities corresponding to about 3000 quanta per second can be recorded. Although this is one-tenth the sensitivity of the dark-adapted eye it is still one hundred times or more the sensitivity required for most light scattering measurements.

Due to difficulties in manufacture, the photomultiplier tubes vary considerably in their sensitivity and dark current. It is desirable to choose a particular phototube which is low in dark current and high in sensitivity. The manufacturer has already done this for the RCA 1P21 photomultiplier tubes. Moisture in the scattering chamber can increase considerably the dark current. It is desirable, therefore, to have a drying agent in the light scattering chamber at all times excepting when the instrument is in actual use.

The photocurrent may be measured directly on a high-sensitivity moving coil galvanometer (Brice, Halwar, and Speiser, 1950) or may be amplified with an electrometer tube and recorded on a microammeter or on a recording potentiometer (Oster, 1953). The amplification procedure is preferable in that the recording instrument is not subject to external mechanical vibrations and, furthermore, responds quickly to sudden changes in intensity as is required for individual bacterial counting. During the course of an experiment it is usually necessary to measure scattered light of a wide range of intensities. It is therefore desirable to be able to vary the sensitivity of the instrument over a ten-thousand-fold range accurately in steps of ten. Angular measurements are made by moving the photomultiplier tube about the center of the sample cell. If the light beam is reduced in width by a nosepiece and if a nosepiece is also placed before the detector a cylindrical sample cell may be used. Narrow beams, small compared with the radius of curvature of the cell, will not be appreciably distorted by the cell.

In Fig. 7 is shown diagrammatically the optical system of the Aminco instrument. The base of the lamp has three positioning screws with which to alter the position of the lamp to obtain maximum light intensity on the sample. The lamp is enclosed in a housing which serves both as a reflector and to help dissipate heat via a chimney. A metal shield further protects the sample chamber from the heat of the lamp. A removable light stop is located next to the lamp. A shutter on a spring may be opened momentarily or locked in the open position. The shutter is released by twisting the knurled cap back to its original direction, whereupon it will spring up to its normally closed position.

A total of six optical components may be added at the filter positions shown in Fig. 7. The filter holders are all of the same dimensions and the optical element is inserted in the 16-mm holes provided and is held in place by retainer rings. Among the optical elements employed for various measurements are polarizers for polarizing the light vertically and horizontally, an opal glass diffusing plate, converging lens of 40-mm focal length, neutral filters, yellow glass filter, and a heat filter.

Places for five filters in the form of a rotating turret are provided for at position 2. Five colored glass filters which isolate the lines of mercury are

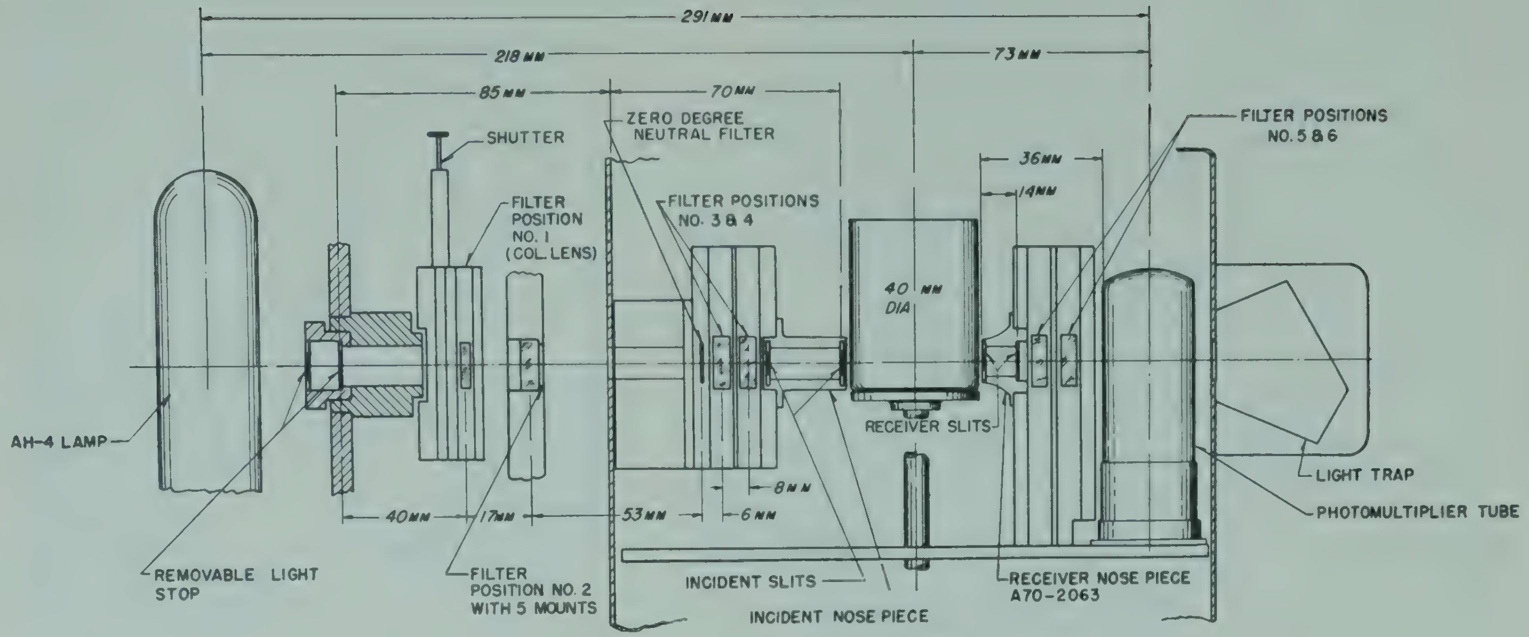
291 MM
218 MM
73 MM
85 MM
70 MM
36 MM
14 MM
ZERO DEGREE NEUTRAL FILTER
SHUTTER
FILTER POSITION NO. 1 (COL. LENS)
FILTER POSITIONS NO. 3 & 4
FILTER POSITIONS NO. 5 & 6
40 MM DIA
AH-4 LAMP
RECEIVER SLITS
LIGHT TRAP
PHOTOMULTIPLIER TUBE
8 MM
40 MM
17 MM
53 MM
6 MM
REMOVABLE LIGHT STOP
FILTER POSITION NO. 2 WITH 5 MOUNTS
INCIDENT SLITS
INCIDENT NOSE PIECE
RECEIVER NOSE PIECE A70-2063

FIG. 7. Optical system (Oster, 1953).

inserted in the turret, so that monochromatic light of any one of these wave lengths is quickly obtained.

When the phototube in its housing is at less than 25° (all angles are taken with respect to the transmitted beam), a mechanism attached to the movement of the phototube automatically places a dark neutral filter (density greater than 4) in the path of the transmitted beam. This dense filter protects the phototube from direct transmitted light at 0°. When not in position, the neutral filter remains below the optical axis. The filter may be removed for small-angle observations or for transmittance measurements of samples of very high absorbances.

The beam is collimated by means of the incident nosepiece, which consists of two slits in a cylinder. Two incident nosepieces may be used. The larger nosepiece has a rectangular entrance slit, 2.5 × 5.0 mm, and rectangular exit slit, 3.8 × 7.5 mm, the distance between the slits being 24.0 mm. The smaller nosepiece has two square slits 2.8 mm on an edge, 15.0 mm apart. The receiver nosepiece on the phototube housing has two equal slits 2.5 mm high and 1.0 mm broad, and 15.0 mm apart. These nosepieces fit into the standard 16-mm holes and have a positioning pin and groove to keep the long axis of the slit in the vertical position. With the smaller incident nosepiece the angular divergence is about 1°. Convergent light at the center of the sample is achieved by placing the converging lens at position 3. By placing the lens at position 1, a three- to fourfold increase in intensity in the sample chamber is achieved.

A light trap is placed outside the sample chamber at the 0° position. It may be removed for visual alignment of the optical system. Stray radiation is further reduced by blackening the folder holders, nosepieces, etc., and by coating the sample chamber surfaces with a black rayon flocking. With the chamber lid and shutter closed, the instrument at its highest sensitivity does not record the presence of any stray radiation even in a brightly lit room. The phototube is rotated manually from outside the sample chamber. The detector may be rotated from 0° to 147° in a horizontal plane about the transmitted beam, the angles being read off on a wheel marked in degrees. A photograph of the instrument is given in Fig. 8. The over-all length of the instrument, including the electrical system, is 24 inches.

When only small amounts of solution are available (of the order of 1 ml) a small test tube containing the sample may be supported in an upright position at the center of the cylinder and surrounded by the solvent of the solution to be examined. Temperature control from an external constant temperature bath can also be achieved for this cell (Cohen, Hiskey, and Oster, 1954). A goniometer has been constructed for photometric measurements of flat samples as is done in diffraction experiments. The sample, 20-mm square or larger, is held in a clip and may be oriented at various

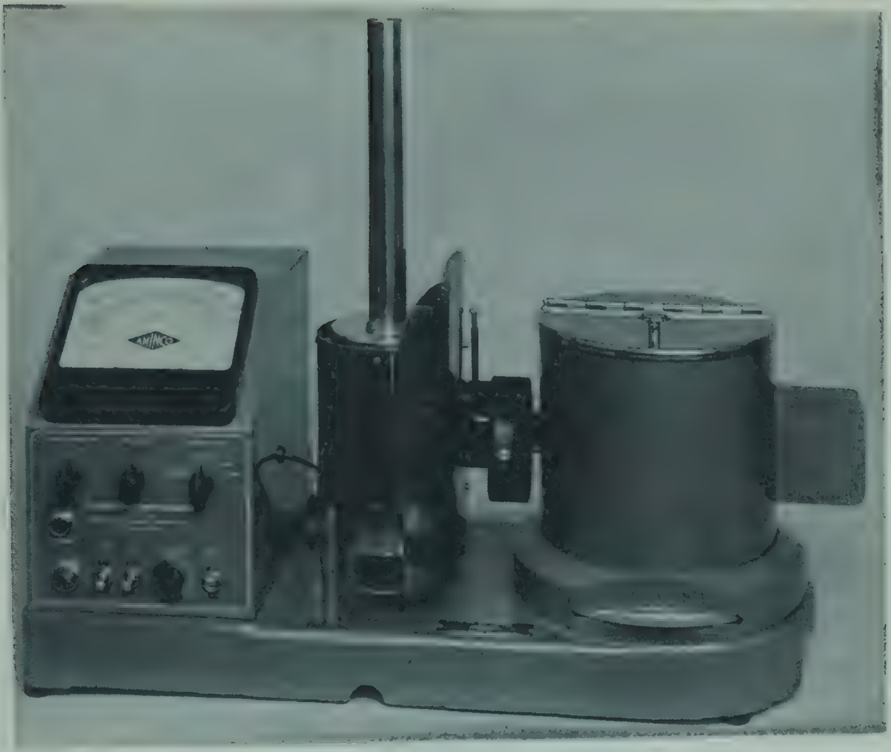

FIG. 8. Light scattering photometer (Oster, 1953).

angles with respect to the incident beam, the angles being given on a graduated scale.

IV. Experimental Procedures

The solutions to be used for quantitative light scattering determinations should be scrupulously free of dust and other extraneous particulate matter such as denatured protein. This is particularly important if the solution exhibits a low turbidity (less than 10^{-2} cm^{-1}). Dust can be removed by passing the solution through a fine sintered glass filter. There is a danger, however, that the solute may be adsorbed to the filter or be entrapped in the pores. This is particularly the case for highly elongated molecules. Easily denatured proteins such as ovalbumin will suffer radical changes when subjected to filtration. A more suitable procedure is to clarify the solution by centrifugation. Particulate matter can usually be removed by centrifuging at from 5,000 to 10,000 r.p.m. For certain solutions such as those of desoxyribose nucleic acid which are highly viscous the centrifugation should be carried out over several hours.

A solution can be tested visually as to whether it is sufficiently clarified. If the solution is illuminated by a bright light such as a microscope lamp and viewed at an angle close to the transmitted beam (it is well to keep only one eye open for these observations), dust in the solution is readily discernible. Observations at right angles are without value since the dust particles are large and therefore scatter strongly in forward directions.

It is generally most convenient to start with a relatively high concentration of solution and then to carry out serial dilutions with solvent which also has been clarified. A starting solution should have a turbidity in the neighborhood of $10^{-2} - 10^{-3}$ cm^{-1}. For the special case in which transmission measurements are to be carried out, the turbidity must be in the neighborhood of 10^{-1} cm^{-1} or greater.

The absolute turbidity of the solution can be determined by comparing the intensity of scattering at 90° with that for some reference solution and then applying the appropriate dissymmetry corrections as discussed in Section II, 3 above. A convenient reference solution is any solution which has a high enough turbidity so that it can be determined by transmission and yet has a dissymmetry close to unity. Ludox, a commercially available stable silica suspension, is ideal for this purpose (Tietze and Neurath, 1952; Mommaerts, 1952; Oster, 1952). The deflection of the meter of the light scattering instrument which is proportional to the light intensity is read at zero degrees for the sample cell containing distilled water. The water is now replaced by the Ludox solution and a reading is taken. The turbidity of the Ludox suspension is then given by Eq. 1 where I/I_0 is the ratio of the readings for Ludox and for water, respectively. For the cell illustrated in Fig. 7 the path length l is 4.0 cm. The photomultiplier tube is now moved to 90° and the meter deflection now corresponds to the turbidity obtained by the transmission method. The deflections at 90° for the solution under investigation are then compared with the reference solution.

Angular measurements should be carried out for a series of dilutions and the values extrapolated to infinite dilution. The pure solvent will generally have a turbidity in the neighborhood of 10^{-4} cm^{-1}. It is not necessary to correct for the contribution due to the scattering by the solvent unless one is dealing with solutions with turbidities comparable with this low value.

Light scattering measurements can also be carried out on colored solutions but the scattering data must be corrected for the light absorption which, necessarily, is determined by the geometry of the sample cell (Brice, Nutting, and Halwer, 1953). If the solution exhibits an intensity of fluorescence comparable with the intensity of scattering it is desirable to use an incident light of longer wave length even at the expense of obtaining much more feeble light scattering as shown by the Rayleigh's inverse-fourth power relation (Eq. 1).

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Chapter 3

ABSORPTION SPECTROSCOPY

C. F. Hiskey

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I. Introduction

1. Purpose

The purpose of this chapter is to review for the biochemist and the analyst those fundamental principles of spectrophotometry most necessary to an understanding of this method of measurement. By illustrating their application to the multitude of problems which may be solved by a spectrophotometric approach it is hoped that an appreciation of the utility of this analytical area will be developed. Inasmuch as many notable advances have been made in the past decade in the field of spectrophotometry, the main emphasis in this article will be upon these newer areas of development.

The principal concern of spectrophotometry is with measurements of the relative ability of a substance to absorb light of any given wave length but particularly in the wave length region between 0.2 and 10 microns. The main reason for making such measurements is their usefulness in the rapid chemical analysis of solutions and other materials. In the visible spectrum this type of analysis is often referred to as colorimetry. Apart from analysis, spectrophotometry may be used as a general research tool in a great many fundamental investigations. It is the intent here to develop not only the analytical applications of spectrophotometry, but also to demonstrate its application in the solution of a variety of fundamental problems.

2. Interaction of Radiant Energy with Matter

The known properties of light are quite successfully described with the aid of two quite different models. With one of these models, light is regarded as being composed of wave trains; in the other it is regarded as a collection of particles.

The first of these models, which underlies the electromagnetic theory of radiation, allows a detailed description of such phenomena as reflection, refraction, dispersion, scattering, interference, and diffraction. The assumptions of this theory are:

a) That radiation consists of wave motion with electric and magnetic vectors at right angles to each other and to the direction of light,

b) That matter is composed of particles, electrical in character and capable of having an electrical dipole induced in them as a result of interaction with the light wave.

To account for the actual absorption or emission of light, however, the second model is needed. This model underlies quantum and wave mechanical light theory. It allows a description of such processes as emission of light from a black body radiator, the photoelectric effect, the absorption and emission of light of discrete wave lengths by atoms and molecules, to name a few of the more important ones. In all of these processes if any light energy is absorbed, it is absorbed as a "chunk" or quantum.

To understand in a preliminary way the various ways in which light interacts with matter, particularly in those areas of concern to absorption spectroscopy, it will be useful to review a number of elementary concepts about waves.

Light is known to have a velocity in vacua of about 3 × 10^{10} centimeters per second. It is said to be monochromatic when the bundle of waves all possess identical wave lengths. Any individual light wave may be represented as a regular fluctuation of its electrical component, Fig. 1. In such a plot the maximum displacement from the base line is referred to as the amplitude, γ. The point on the wave of maximum displacement is referred to as the antinode while that point where the wave crosses the base line is called the node. The distance separating any two nodes or antinodes on the same side of the base line is called the wave length. The units of length commonly associated with light waves of interest to the spectroscopist are the micron, $\mu = 10^{-6}$ meter $= 10^{-4}$ cm; the millimicron, $m\mu = 10^{-9}$ meter $= 10^{-7}$ cm and the angstrom, $\text{Å} = 10^{-10}$ meter $= 10^{-8}$ cm.

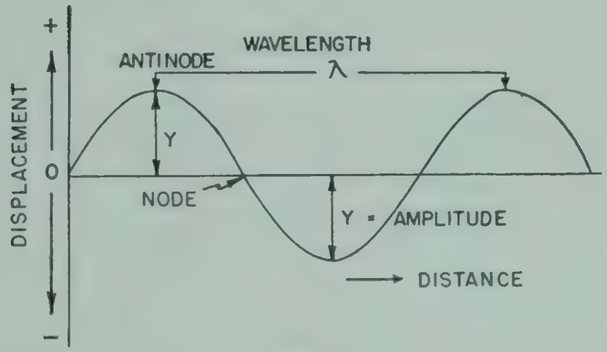

FIG. 1

Associated with the idea of wave length is that of frequency. The frequency of any light wave will be equal to the number of times it goes through a complete oscillation each second. The frequency, ν, is related to wave length λ and the velocity c of light by the relation $\nu = \frac{c}{\lambda}$.

It is frequently desirable to use a quantity proportional to the frequency but smaller than it by 3 × 10^{10}. This quantity called the wave number is equal to the reciprocal of the wave length, i.e., $\frac{1}{\lambda}$.

The energy associated with a light beam is specified by the amplitude of the wave and by its frequency. Thus for a given frequency the intensity of the light beam is proportional to the square of the amplitude, γ, and is directly related to the number of wave trains or quanta passing per second through any plane at right angles to the beam. It is analogous to the number of amperes flowing in an electrical circuit.

On the other hand, the frequency of the electromagnetic oscillation is a force term. It is analogous to the potential difference in electricity. In general the greater the frequency of oscillation, the greater the energy associated with the individual wave train or photon. There is, consequently, a unit of light energy termed the einstein representing the quantity associated with Avogadro's number of photons of a given frequency. This unit of energy was initially defined by the Einstein photochemical equivalence relation, i.e.,

TABLE I

Useful Constants

1μ	$= 10^{-4}$ cm; 1 mμ = 10^{-7} cm; 1 Å = 10^{-8} cm.
c	Velocity of light *in vacuo* = 2.99776 × 10^{10} cm per sec
h	Planck's constant = 6.624 × 10^{-27} erg-sec
N	Avogadro's number = 6.023 × 10^{23}
e	Electronic charge = 4.803 × 10^{10} absolute
	Electrostatic units = 1.602 × 10^{-20} absolute
	Electromagnetic units
	Electron mass = 9.11 × 10^{-28} g
1 watt	= 1 joule per sec = 10^7 ergs per sec
1 calorie	= 4.185 joules = 4.185 × 10^7 ergs
1 ev	= 23,055 calories/mole

$$E = Nh\nu = Nev. \tag{1}$$

N is Avogadro's number, h is Planck's proportionality constant. The einstein may also be expressed in terms of the number of electron volts, v, associated with the photon and the electronic charge e. The values of the constants referred to above as well as some others useful in spectrophotometry are given in Table I.

Using Eq. 1 it is an easy matter to compute the amount of energy expressed either as kilogram calories or electron-volts associated with any given frequency. To do this we substitute constants from Table I into Eq. 1 as follows:

$$E = N\frac{hc}{\lambda} = \frac{(6.023 \times 10^{23})(6.624 \times 10^{-27})(3.0 \times 10^{10})}{(4.185 \times 10^{10})(10^{-7})(\lambda \text{ in } m\mu)} = \frac{28{,}579}{\lambda \text{ (in } m\mu)}$$

Thus we see that 28,579 divided by the wave length in millimicrons gives us the kilogram calories associated with Avogadro's number of quanta having that particular wave length. 4.185×10^{10} equals the number of ergs in a kilogram calorie while 10^{-7} is the conversion term for mμ into centimeters.

It is also useful to compute the number of electron volts (i.e. the voltage through which the electronic charge, e, must be accelerated to gain an equivalent amount of energy) associated with an einstein. The expression here may be shown to be

$$\text{Electron volts} = \frac{1239.5}{\lambda(\text{in } m\mu)}$$

The energy possessed by yellow sodium light (589 mμ) is thus seen to equal 48.5 kcals or 2.1 electron volts. A nomograph calculated from these relations is provided in Fig. 2. It covers the wave length interval between 100 and 1000 mμ.

It is readily seen that with increase in frequency the energy associated with a quantum increases. Since the known wave length interval of the electromagnetic spectrum extends from the longest radio waves (1000 meters) to the shortest gamma rays (10^{-13} meters) the variation of quantum energy within this interval varies by a factor of 10^{18}. It is therefore to be expected that the effects produced by the interaction of various quanta with matter will alter profoundly with wave length. The nature of the various interaction processes will now be considered.

The vibrations of electromagnetic waves are analogous to those of an alternating current. When such waves pass through matter they induce electrical oscillations in the atoms and molecules composing matter. In turn the molecules are assemblages of electrons and atoms bound together by electrical forces. Such assemblages have natural periods of vibration not unlike those of a piano string or a pendulum, and as the waves pass through matter the electrical structure of the atoms and molecules is distorted. This

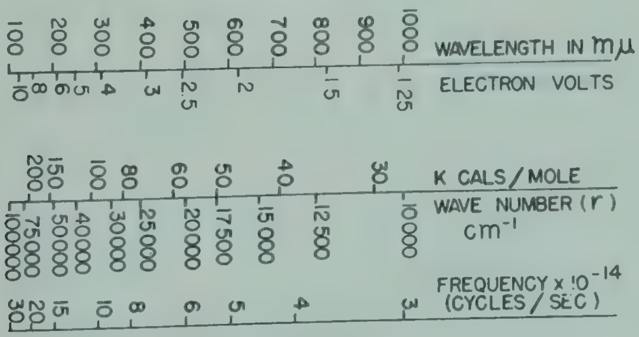

FIG. 2

distortion fluctuates with the amplitude of the exciting waves; it is presumed to have the same frequency as the exciting wave but to lag behind it somewhat.

The extent to which matter interacts with the wave depends upon how close the natural periods of vibration of the atomic and molecular structures are to that of the exciting wave, i.e., the closer they are the greater the interaction and vice versa. The duration of these induced oscillations, for visible light, is between 10^{-14} and 10^{-15} seconds providing the frequency is not close to that of the natural frequency. If it is close, then it rises quite sharply to 10^{-8} or 10^{-9} seconds.

The electrical energy stored in the atoms or molecules by interaction with light is given out again as radiation of unchanged frequency, but this radiation interferes with the exciting radiation to produce new waves. The character of these new waves again depends upon how close the exciting frequency is to the natural frequency of the electrons, atoms, or molecules:

a) If these two frequencies are widely separated, then when a beam of light impinges upon matter it will be divided into a reflected ray and a refracted ray. The relative distribution of the light energy between these two components is quantitatively described by equations due to Fresnel. In general it is determined by the angle of incidence of the light beam and the refractive and absorption indices of the material.

b) On the other hand, if the exciting and the natural frequencies are quite close or identical then several different kinds of interactions can occur depending upon whether or not the electrical energy induced in matter is recovered at the same or different frequencies.

As an example of the first of these cases—if mercury vapor even at quite low pressures is illuminated by light with a wave length equal to 253.652 mμ, it will be found that the light beam does not penetrate very far into the gas but is re-radiated in all directions. If the vapor pressure is great enough so that the individual atoms are separated by distances which are small relative to the wave length of the incident beam, then the re-radiated waves combine to give what has been termed selective reflection. That is, the surface of the vapor behaves as a perfect reflector for this one wave length. For others, it is transparent.

The surfaces of crystals of many dyes show this same property. A well known example is that of methyl violet crystallized on the bacteriologist's microscope slide.

As an illustration of the second of these cases, i.e., where the exciting and natural frequencies are identical or nearly so, the following is pertinent:

Under certain conditions when light energy is absorbed by matter, the energy is converted rapidly into other forms, usually in the order of 10^{-8} to 10^{-9} seconds. This energy is radiated sometimes as fluorescent light in which it is not of the same frequency as the incident radiation. It may also go into chemical energy by dissociating molecules and producing various chemical transformations. Finally, it may become heat energy by being converted into translational, vibrational, or rotational energy. This last is the process with which we are largely concerned in absorption spectrophotometry.

The effects of energy absorbed in this way on the atom and molecule vary considerably with the energy associated with the individual quantum of light. For example, when the wave length of the light is of the order of 50 to 100 microns, i.e., in the far infrared, the excitation produced in the molecule will alter its period of rotation. In the near infrared region, i.e., 1 to 10 microns, absorption of radiant energy affects the vibrational states of atoms in the molecule. In the ultraviolet or visible region where the wave lengths are of the order of 0.2 to 0.8 microns, excitation of the electrons in the outer shells of the atom occur. With even shorter radiations such as x-rays, i.e., 10 to 1 mμ, excitation of the inner electron shell of the atom occurs. Finally for gamma rays, that is a hundredth of a mμ and less, the interaction is associated with changes in the energy level of the nuclei of atoms themselves, and thus involve transformations of these nuclei.

II. Factors Involved in Taking Data

1. Light Losses Due to Reflection and Scattering

When a beam of monochromatic light falls on a homogeneous layer of a substance a part of the light is reflected, or scattered, a part is absorbed and the remainder is transmitted. If the intensity of our initial beam is represented by I, then we may summarize what has been said above by writing

$$I_R + I_A + I_T = I. \tag{2}$$

where the subscripts R, A and T refer to reflected, absorbed, and transmitted light components.

When I_R is properly compensated for, our equation becomes

$$I_0 = I_A + I_T = I - I_R\,. \tag{3}$$

In Eq. 3 I_0 would represent the monochromatic light energy emerging from some reference absorption cell, containing all the chemical ingredients of our reaction except the light absorbing substance of interest. I_T would represent the light emerging from the same or identical absorption cell containing the absorber in addition to the other ingredients. I_T/I_0 will be the fraction of light energy transmitted or the transmittance.

In spectrophotometry we are primarily interested in I_A, but it will be useful to review the factors that lead to light scattering and reflection so that if reflective losses cannot be sufficiently minimized or avoided altogether, at least proper compensation or evaluation of this term can be effected.

Whenever a ray of light passes from a medium of one density into a medium of a different density, reflection occurs at the interfacial boundary. There are two principal types of reflection, i.e., specular and diffuse. Specular reflection is given by perfectly polished glasses, metals, or other mirror-like surfaces. Diffuse reflection occurs when a light beam passes through a suspension of particles in some continuous medium; the term "light scattering" is generally applied to this kind of reflection. Although both types occur from a common cause, the mathematical equations describing such reflections differ considerably. Consequently, we will consider each type of reflection in turn.

a. Reflection at Plane Surfaces

Suppose that a beam of monochromatic light of intensity I were to fall on a transparent glass surface, as in Fig. 3, at some angle i relative to the perpendicular line drawn to the glass surface. A portion of the light of intensity I_R would be reflected off the surface at the same angle to the perpendicular as the incident ray, and another fraction I_T would be refracted into the body of the glass. The angle which this refracted ray takes to the perpendicular would be equal to r and is given by Snell's law

$$n \sin i = n' \sin r. \tag{4}$$

The letters, n and n', in Eq. 4 refer to the refractive indices of the respective media. In the case we are considering n and n' would be equal to unity and 1.50 respectively. It can be seen that if the angle i is equal to zero then $\sin i$ would be zero and r also would be equal to zero. Therefore, perpendicu-

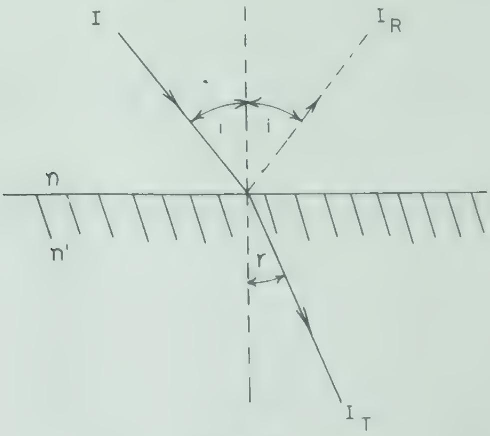

FIG. 3

lar rays will pass directly through the interface. When i equals 90° then sin i would equal unity and sin r would equal 0.66, corresponding to an angle r equal to about 41°48′. Thus even though the incident beam approaches the grazing angle, the angle of the refracted ray will not become greater than 41°48′.

The ratio of the sines of the angles as in Eq. 5 equals the ratio of the

$$\frac{\sin i}{\sin r} = \frac{n'}{n} \tag{5}$$

refractive indices of the two media. In the case under discussion, since n equals unity the ratio of these two sines gives us the index of refraction of the glass directly.

Suppose, however, that instead of using monochromatic light we used a pencil of white light. We would then observe that the refracted ray was spread out into a spectrum. This arises from the fact that most glasses show an increase in the refractive index as the wave length of the light decreases (illustrated in Fig. 4). If n' increases then sin r must decrease. Therefore, the refracted ray will be bent more for the shorter waves than for the longer waves. This well known phenomena is called "dispersion." It is used extensively in the design of prism monochromators.

It will be of interest to us to consider how I_R in Fig. 3 varies as a function of the angle of incidence. For this purpose we will once more assume that we are working with monochromatic light. The relation governing the fraction of light reflected from a glass surface was derived first by Fresnel and is given for unpolarized light by Eq. 6.

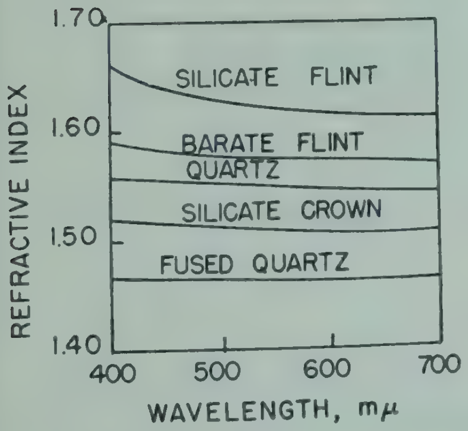

FIG. 4

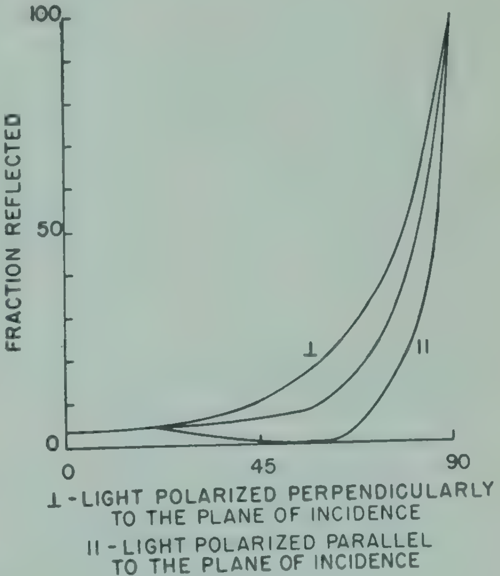

FIG. 5

$$I_R/I = \frac{1}{2}\left(\frac{\sin^2(i - r)}{\sin^2(i + r)} + \frac{\tan^2(i - r)}{\tan^2(i + r)}\right) \tag{6}$$

It will be seen that Eq. 6 is composed of a sine term and a tangent term. These two terms refer respectively to light polarized perpendicular and parallel to the plane of incidence. One half the sum of these two terms gives us the relation for unpolarized light.

In Fig. 5 the variation of reflectance as a function of the angle of incidence is plotted. The uppermost curve is the sine term while the lowest curve is the tangent term. The middle curve corresponds to the expression given in Eq. 6. Examination of Fig. 5 reveals that when the light beam is normal to the plane of the glass surface then both the parallel and the perpendicular components are reflected in equal amounts, indicating that the reflected ray is not polarized. As the angle of incidence departs from the normal a variation in the reflectance term for the two components is observed, which reaches a maximum at about 55°. When the sum of i plus r equals 90° then the denominator of the tangent term becomes infinity and no reflection of the polarized light parallel to the plane of incidence occurs. The angle i at which this occurs is termed the polarizing angle and may be calculated with Brewster's relation (Eq. 7).

$$\tan i = \frac{n'}{n} \tag{7}$$

Thus it is apparent that when the incident angle equals the polarizing angle only one of the polarized components is reflected. As the grazing angle is approached the reflection of the two polarized components again becomes identical. The important fact which emerges here is that whenever reflection occurs from a glass surface at any angle other than the normal one, both the refracted and the reflected rays will be partly polarized.

If Snell's law is substituted into Eq. 6 and the limitation is imposed that the angle of incidence equals zero, then it can be shown that the fraction of reflected light reduces to a very simple expression. Inserting

$$I_r/I = \left(\frac{n - n'}{n + n'}\right)^2 \tag{8}$$

values of 1.0 and 1.5 for the refractive indices of air (n) and glass (n'), respectively, into Eq. 8 reduces it to

$$I_r/I = \left(\frac{1 - 1.5}{11.5}\right)^2 = .04$$

This means that 4 % of the intensity of the incident beam will be found as a reflected ray while 96 % will be transmitted through the air-glass interface when the light beam strikes it at a 90° angle.

The expression also indicates that the greater the difference between n and n' the greater will be the reflectance. By coating glass surfaces with substances whose refractive indices are intermediate to those of the air and the glass a substantial reduction in the reflective losses may be effected as the reader may easily demonstrate. These facts, however, only serve to show the analyst how critical it is to keep the outer and inner surfaces of the absorption cell in a constant state of cleanliness. Failure to do so will alter the reflectance and cause serious errors in the absorbance measurements. To illustrate the problem of reflectance losses, two situations will now be considered. In the first of these reflectance losses will be computed for a beam of light passing through an absorption cell containing water only. In the second, the same reflectance losses will be computed assuming that the absorption cell contains an aqueous solution of some colored dye whose transmission is only 10%.

In the first case, as the beam of light passes through the cell it suffers reflection losses at the four interfaces but it is not otherwise affected. The intensity of the light beam after each of these four reflection stages is as follows:

After passage through the:	*Intensity of ray equals:*
First air-glass interface	$I(1 - .04) = .96I$
Plus first glass-water interface	$I(1 - .04)(1 - .0036) = .956I$
Plus second water-glass interface	$I(1 - .04)(1 - .0036)^2 = .953I$
Plus second glass-air interface	$I(1 - .04)^2(1 - .0036)^2 = .915I$

The assumption in these calculations is that the refractive index of water is 1.33. It is seen, therefore, that even in this relatively transparent system the intensity of the light beam is diminished 8.5% before it emerges from the cell. At every air-glass interface the intensity of the transmitted beam is reduced by 4% while at the glass-water interfaces the intensity is reduced by 0.36%. Except in the case of the first interface, these per cent reductions are not of the incidence beams but on the residue which has been transmitted up to that particular point.

In our second case, where the absorption cell contains a solution whose transmission is only 10% of the light entering, the following intensities are observed at the several interfacial boundaries:

After passage through the:	*Intensity of ray equals:*
Air-glass interface	$I(1 - .04) = .96I$
Plus glass-solution interface	$I(1 - .04)(1 - .0036) = .956I$
Plus solution	$I(1 - .04)(1 - .0036)(.1) = .0956I$
Plus solution-glass interface	$I(1 - .04)(1 - .0036)^2(.1) = .0953I$
Plus glass-air interface	$I(1 - .04)^2(1 - .0036)^2(.1) = .0915I$

These intensity relations are diagrammed in Fig. 6. In general it is seen that they are identical as the beam passes through the first two interfaces, after which their courses diverge. The important element in this figure is that the absolute amount of reflected light is greater in the case where water was present in the absorption cell instead of the colored solution.

But this does not affect our measurement of the fraction of light transmitted, since that ratio will equal $\frac{0.0915I}{0.915I} = 0.1$ or 10%. In other words, because each of these percentage changes applies to the residue, they all cancel out in the ratio transmitted except for the actual ratio.

b. Diffuse Reflection

When a beam of light passes through a medium containing suspended particles a lateral scattering of light will be observed at all angles to the incident beam. If white light is used, and the particles are relatively small compared to the wave length of the light, the scattered light will take on a bluish character while the transmitted light becomes increasingly yellow or orange. Such effects may be conveniently observed by putting a few drops of milk into a beaker of water meanwhile either viewing the scattered light from the beaker at right angles to the beam or looking directly at the transmitted beam through the beaker. Extensive studies of the quantitative aspects of such light scattering by small particles have been made and are reviewed in Chapter 2.

The important aspects of small particle scattering to the analyst are these: a) the amount of light scattered is very dependent upon the wave length of light employed; b) the fraction of light scattered is a very high power of the radius of the scattering particles. It has been stated previously that the light scattered by a dilute milk suspension had a bluish color. This is an indication that light of shorter wave length is scattered more than light of longer wave length. Lord Rayleigh showed in 1871 that such scattering was proportional to $(1/\lambda)^4$. Light at, let us say, a wave length of 800 mμ has a wave length twice as great as that of violet light at 400 mμ. One can therefore expect that the blue light would be scattered with 2^4 or 16 times the intensity of the red light. Recognition of this relationship often makes it possible to separate from an absorption curve that part which is due to true mo-

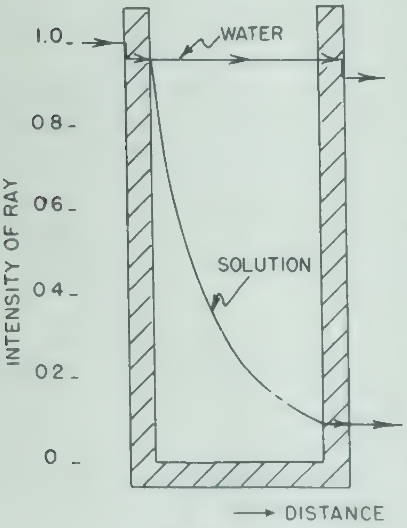

Fig. 6

lecular absorption and that part which is due to scattering. Illustrations of such situations will be given below.

It may also be observed from Lord Rayleigh's equation that light scattering is dependent on the 6th power of the particle radius. This means that for a fixed percentage of suspended matter the fraction of light scattered increases with increasing particle size. Wherever it is possible to remove suspended matter without altering the concentration of substances of analytical interest, it is preferable to do so.

2. The Bouger-Beer or Absorption Law

In 1729 Bouger correctly stated the law relating the light absorption to the path length in a homogeneous absorbing medium; in 1852 Beer demonstrated that varying the concentration of the absorbing substance had the same effect as varying the path length of the light. Figure 7 illustrates a situation where two beams of light of identical frequency and intensity are permitted to fall on two identical absorption cells which contain, respectively, pure solvent and colored solution of concentration c. The fraction of the light transmitted by the solution may be written as I_1/I_0, where the intensity of the light beam emerging from the cell containing solvent and solution are written as I_0 and I_1 respectively. In general, I_1 will be less than I_0 whenever the concentration of the absorber is greater than zero. The fraction of light transmitted by the solution is related to the concentration and path length by the Bouger-Beer relation, viz., Eq. 9. In this

$$I_1/I_0 = 10^{-abc} \tag{9}$$

expression, a is the absorptivity, b is the path length of the light through the solution, and c is the concentration. If b is expressed in centimeters and c in mols per liter then a becomes the molar absorptivity. It is not necessary that c be expressed in mols per liter; it may be expressed in any unit of concentration desired. If this is done, however, the value of the absorptivity will vary from case to case.

For example, if the concentration is expressed in grams per hundred cc then the absorptivity coefficient obtained will equal $aM/10$. Where the units of concentrations are expressed in milligrams per cent the absorptivity obtained will equal 100 Ma.

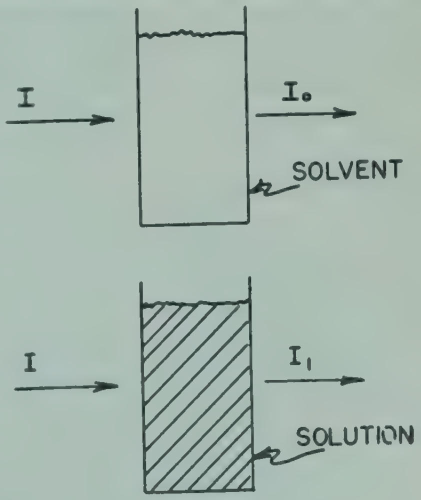

Fig. 7

M in these relations represents the molecular weight. The Bouger-Beer relation is often given in natural logarithms, i.e.

$$I_1/I_0 = e^{-klc}$$

Providing the path length, l, is given in centimeters the absorptivity, k, will be equal to 0.4343 a. By taking negative logarithms of both sides of Eq. 9 it may be transformed into the familiar form (10); A_1 in this expression is termed

$$\log I_0/I_1 = abc_1 = A_1 \tag{10}$$

the absorbance of the solution and is the product of a, b and c_1. This relationship shows us that there is a linear dependence of the absorbance on the concentration.

The significance of the absorptivity may be understood by means of a dimensional analysis of the absorption law. Since the fraction of light transmitted is a dimensionless number, the absorbance A is also dimensionless. On the other hand, b has the dimension of length, i.e. l, while the concentration term c has the dimension of weight per unit volume, i.e. m/l^3. Therefore, the absorptivity, a, must have the dimension l^2/m so that

$$abc = l^2m \times m/l^3 \times l$$

In other words, absorptivity represents area per molecule. It is as though the absorber molecules block the passage of light in proportion to this apparent area. The larger this apparent cross sectional area the greater the probability that a quantum of light will be absorbed by the molecule. The magnitude of a, as a molar absorptivity coefficient will vary from as much as 100,000 for highly colored dyes down to as little as 5 or 10 for some of the faintest colored materials. A substance like permanganate ion, which is considered to be highly colored, has an absorptivity of about 1,000. Quantum mechanical calculations indicate that the absorptivity probably cannot ever be much larger than 10^6.

a. Plotting Spectrophotometric Data

The most useful spectrophotometric data are those showing the light transmitting or absorbing characteristics of a substance as a function of the wave length. For each molecular species there is a characteristic pattern of such data in the spectral range between the far ultraviolet and the near infrared. This pattern often serves as a qualitative identification for the species and provides an indication of the binding energies of the electrons in it.

There are a variety of methods commonly employed in the presentation of such data. These will now be illustrated for three solutions of oxyhemoglobin in which the concentrations have been varied in the ratio of 1:2:4.

In Fig. 8 the per cent transmittance has been plotted as a function of the wave length in the 400 to 600 mμ interval. The numbers associated with the curves indicate the relative concentrations of the solutions employed in obtaining each set of data. It is evident that the more dilute solutions have greater transmittancies at every wave length. The data also show very little transmittance below 440 mμ and transmission minima at 542 and 576 mμ.

Spectral data may also be plotted in terms of per cent absorption, i.e., 100 minus % transmission. This is a more useful and in general a less confusing way to look at spectral data because the wave length intervals under the curve are the ones for which light interaction with the molecular species in solution occurs. As can be seen by reference to Fig. 9, such a plot rotates the transmittance curve through 180°. The transmission minima at 542 and 576 mμ now become absorption peaks at those same wave lengths.

A third way of plotting spectral data, Fig. 10, is in terms of the absorbance, i.e., log I_0/I. The advantage of the logarithmic plot is that a straight

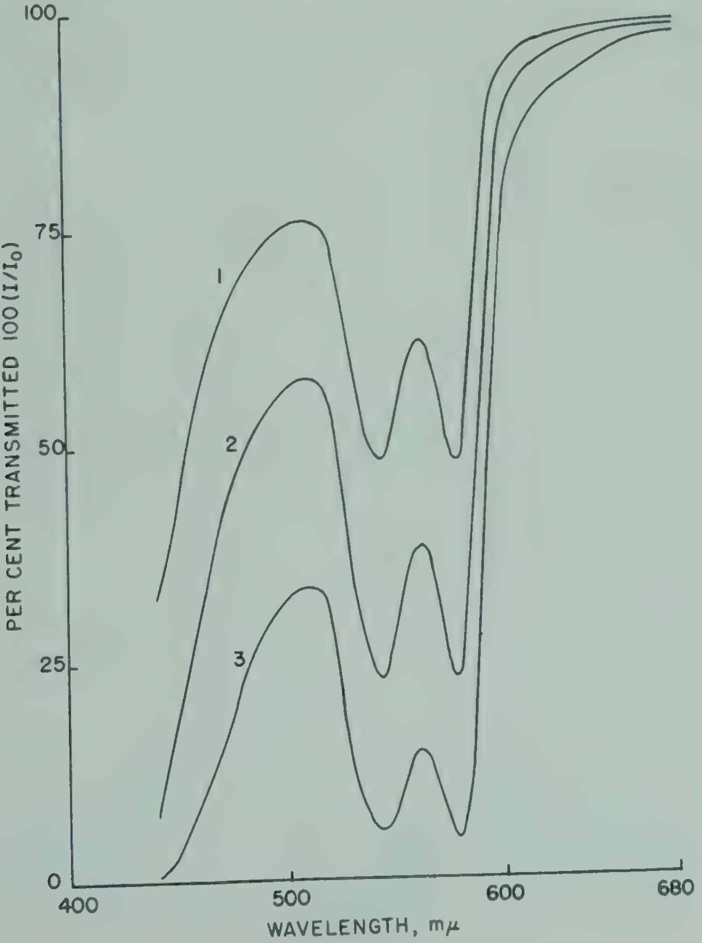

Fig. 8

line relation with the concentration prevails. Therefore, the spectra of oxyhemoglobin of increasing concentration are simple multiples of the lowest concentration curve. Consequently, at any wave length the height of these curves is in a 1:2:4 relation.

If the lowest spectral curve is subtracted from the middle spectral curve at each wave length and the differences plotted, a curve identical to the first curve will be obtained. This is to be expected because the concentration difference between the two is identical with the concentration of the lesser. From these graphs it is also evident that the most sensitive plot of the absorbance as a function of the concentration is made when we are working at an absorption peak. This is always the wave length to be used in simple analytical cases.

Another type of plot of great usefulness in the qualitative identification and comparison of curves is the log log plot. Taking the logarithm of both

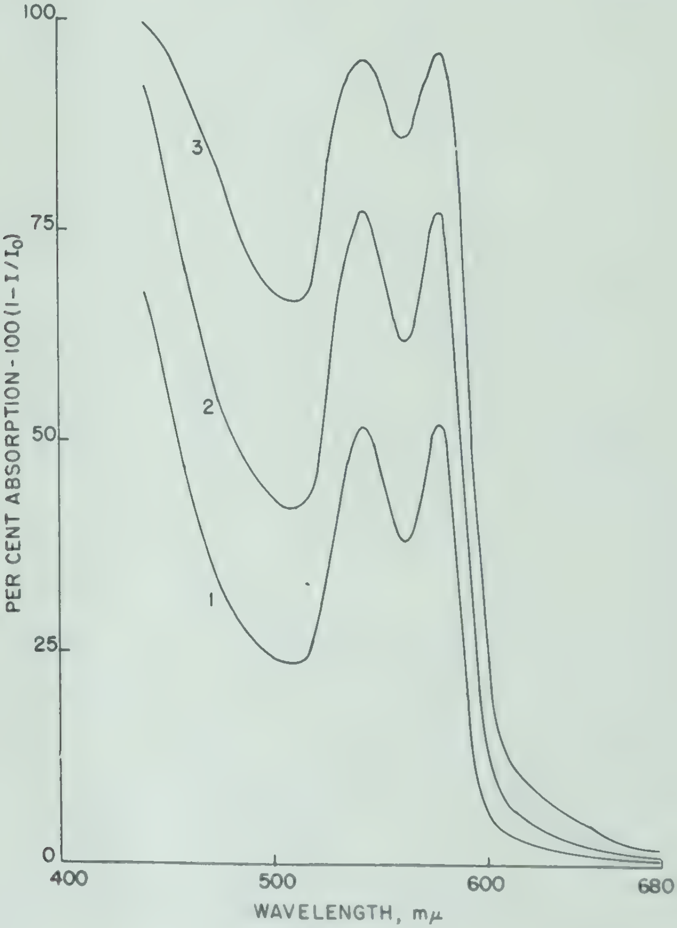

FIG. 9

sides of Eq. 10 there is obtained

$$\log \log I_0/I = \log A = \log a + \log b + \log c. \qquad (11)$$

For any given solution and absorption cell, log b and log c will be independent of the wave length employed. This is not the case with log a. This term varies with wave length and therefore so will log A. Log A will thus be composed of a variable part and two constant parts; the constant parts will determine the lateral displacement of the curve along the ordinate but not otherwise affect it.

The oxyhemoglobin curves are replotted in this manner in Fig. 11, to illustrate the method under discussion. It is seen that all the curves are identical in shape and are only displaced from each other by the variation of the concentration term. By vertical displacement of the curves relative to the lowest, they can be made to coincide at every point. This method of

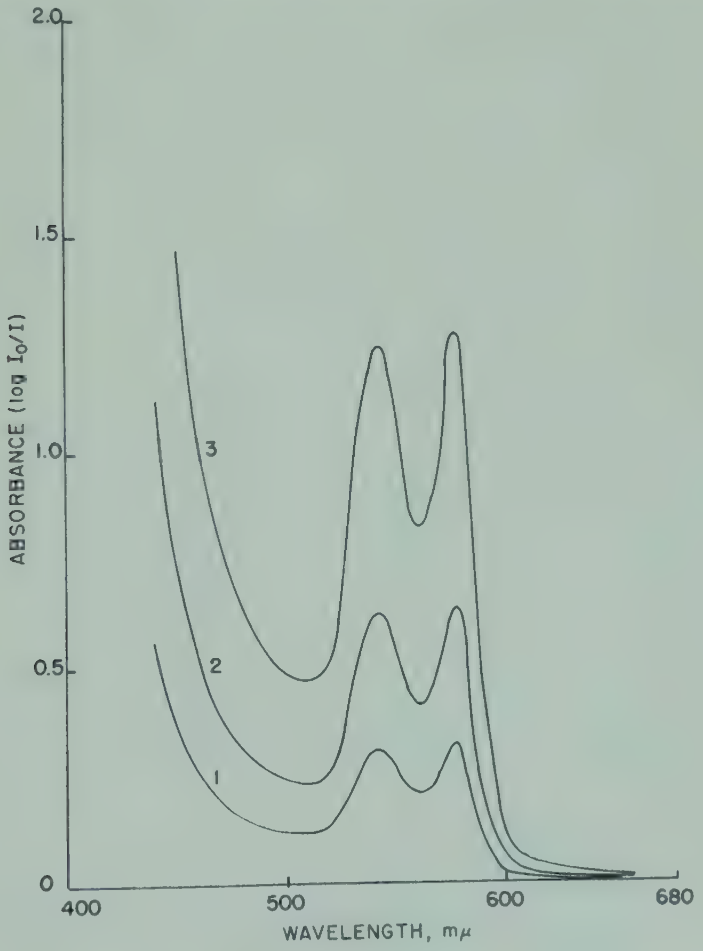

FIG. 10

qualitative identification is not possible with any other type of plot. A recent description of this method has been given by Shurcliff and Stearns (1949).

For the purpose of making a chemical analysis the relationship between the concentration of the absorber and the light transmission properties of the solution must be known. To accomplish this one usually proceeds in the following manner. Having determined the absorption spectrum of the substance whose concentration is to be determined a plot may be made of the absorbance as a function of concentration of a group of standard solutions. When this is done a straight line relationship will usually be observed providing there are no apparent deviations from the absorption law. This straight line relationship makes interpolation between points a relatively easy matter. After determining the absorbance of the unknown solution it is only a matter of seconds to translate that into a concentration value.

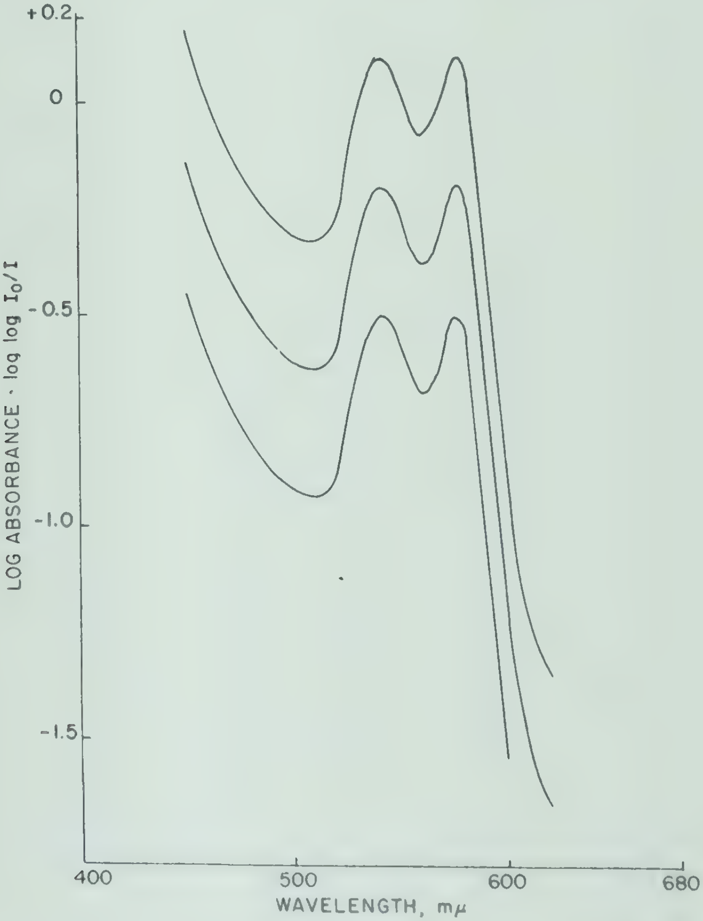

FIG. 11

A plot of the per cent transmittance or of the per cent absorbed as a function of the concentration is less frequently used because of the curvature of these lines.

Calibration curves such as that mentioned above will usually be made at the wave length of the absorption peak. Here a maximum instrument response is obtained for a unit concentration change, and therefore the instrument is most sensitive at this point. In addition, the absorption peaks are generally somewhat rounded. This aspect makes the wave length setting a less critical operation. Where peaks are very sharp, making it difficult to set the wave length with the needed precision, a shoulder on the band may be used in preference.

b. Measurement of Relative Transmittance

Relative transmittance measurements are also of great interest to the biochemist. In this method a solution of known concentration is used as the reference standard while the solution of unknown concentration is compared with it. The advantages of this method of approach are those of rapidity in routine chemical control and of enhanced precision derived therefrom. In addition, there are a number of specific problems which require this method of operation for their successful solution; these will be detailed subsequently.

Suppose two solutions have transmittances given as I_1/I_0 and I_2/I_0. The Bouger-Beer relation for these two solutions may be written as in Eq. 12.

$$I_1/I_0 = 10^{-abc_1} \text{ and } I_2/I_0 = 10^{-abc_2} \tag{12}$$

It will be assumed in this problem that c_2 is larger than c_1. After balancing out the dark current, i.e., setting the zero of the transmittance scale, solution c_1 is inserted in the path of the light beam. By adjusting the slit width (Beckman), the galvanometer shunt (Coleman), or the light intensity (Evelyn), the instrument can be made to read 100% transmittance for this solution. To make a relative transmittance measurement, c_2 is now substituted for c_1. Since c_2 is larger than c_1, the intensity of the beam falling on the photocell will be diminished. Consequently, it will be necessary to re-balance the instrument by turning the transmittance dial to a lower value. The relative transmittance is now given by the ratio I_2/I_1 as in Eq. 13.

$$I_2/I_1 = \frac{10^{-abc_2}}{10^{-abc_1}} = 10^{-ab(c_2-c_1)} = 10^{-ab(\Delta c)} \tag{13}$$

It is the concentration difference, Δc, between the solutions which determines the value of I_2/I_1 and not the absolute concentration of either.

This equation for the relative transmittance differs in no way from the original Bouger-Beer relation. It is only another way of writing that relation. If c_1 were given a value of zero then the expression as written in Eq. 13 immediately reduces to the Bouger-Beer relation. We may expect, therefore, that for truly monochromatic light a plot of the relative transmittance function as a function of the wave length, will give absorption curves identical with those which would be obtained from a direct transmittance measurement if equal concentrations are involved. To set up an analytical method with this type of measurement it is necessary that the absorption spectrum be known; then at the wave lengths of maximum absorptivity a plot of the relative transmittance function may be made as a function of the difference of concentration between the reference standard and a series of other solutions. It will be shown in the discussion of errors of analytical methods that this approach is roughly ten to twelve times more precise than the previous method. To achieve this precision, however, it is necessary for the operator to take greater care in his volumetric technique and in his choice of a reaction for analytical use.

3. Validity of the Bouger-Beer Relation

In textbooks of analytical chemistry and in scientific periodicals one often finds statements to the effect that "this system does not obey Beer's law." It would be more correct to say that there is not a linear relation between the apparent absorbance and the concentration. Such apparent deviations from the Bouger-Beer law are often observed but they are not so much a consequence of the failure of the law as they are a failure of the optical or chemical systems to conform to the requirements of the Bouger-Beer relation. The underlying requirements for the absorption law to apply are that (a) monochromatic light be used and (b) the system be composed of randomly oriented molecular or atomic absorbers. Failure to fulfill these requirements often leads to deviations. The factors responsible fall under two general headings: (a) those related to the instrument, that is, optical factors, and (b) those related to the chemical situation.

a. Deviations Due to Optical Factors

(*1*) *The white light problem.* Whenever monochromatic light is contaminated with a white light impurity, absorption law deviations are found. It is quite easy to understand how this comes about. Assume that the intensity of the monochromatic ray is sufficient to generate a photocurrent I_0, while the white light generates i. Let I_1 equal $I_0 + i$. Now if the monochromatic ray has the same wave length as the absorption peak of the substance being analyzed, then it can be seen that it will be relatively much more strongly absorbed than the white radiations. Indeed, as a first approximation, one might term the monochromatic ray as the absorbable light while the white light may be referred to as the unabsorbable light.

To simplify the treatment of this problem, assume that the fraction of the white light absorbed by the chemical system is so small that i remains essentially constant even though the concentration of material in the cuvette is increased to relatively large values. Under these conditions only the intensity of the monochromatic ray will change as the concentration of absorber is increased. A proper application of the absorption law in this case will give us the relation 14.

$$I_1 = I_0 10^{-abc} + i \tag{14}$$

If we examine this relation it is clear that as the concentration of the absorber increases without limit only $I_0 10^{-abc}$ is going to decrease and in the limit it will equal zero. Thus the quantity on the right hand size of the equation will ultimately come to a constant value given by i. This means, therefore, that with increasing concentration the absorbance will at first appear to follow the Bouger-Beer relation but as the concentration increases further it will begin to deviate more and more from the proper linear relation until finally it will curve over to reach a limiting value. In Fig. 12, a plot of this type of deviation is given for the situation where i has been given the relatively large value of 0.1 I_0. Here it can be seen that the maximum absorbance which can ever be apparently achieved is equal to unity. No matter how much the concentration of the solution may be increased this becomes the limiting value for the system. The apparent transmission, on the other hand, can never be less than 10%.

This type of deviation was extremely common before good monochro-

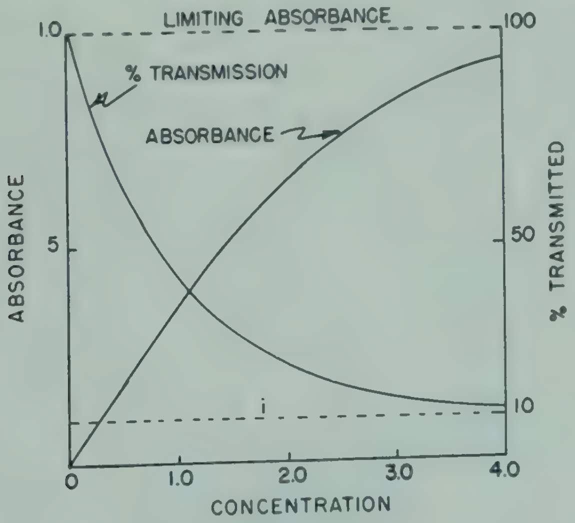

FIG. 12

mators were built. It is still a characteristic of relatively poor instruments. It can, however, be observed with even the best monochromators or spectrophotometers at the extreme ends of the spectrum. Here the beam of the monochromatic ray is relatively attenuated in intensity compared to that of the white light scattered by dust particles and by the edges of the slits. Consequently, the white light can become an important fraction of the total light energy emanating from the instrument. Under such conditions even with a relatively good monochromater sizeable absorption deviations may be anticipated.

(2) *Mixtures of monochromatic rays.* It is now possible to extend the argument made above to the case where two or more monochromatic rays emanate from the slit, each of different intensity and different absorbability. Let us define these two rays as A and B and their intensities as $I_0(A)$ and $I_0(B)$. The ray which is most intensly absorbed by the substance, (A) in solution will be the one which is decreased relative to the other, (B). As a consequence, it will disappear from the composite ray and ultimately it will be only the less absorbable ray which will emerge from the absorption cell.

A plot giving concentration of the chromophor vs. absorbance will show a slope initially determined by the absorptivities of both A and B. As the concentration is further increased, A will be so attenuated that the slope

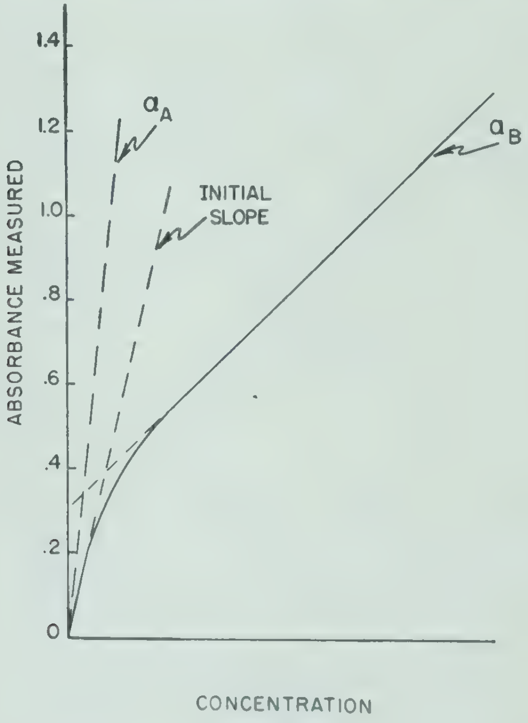

FIG. 13

of the line will gradually shift until it approaches that of the absorptivity of B. Such a situation is diagrammed in Fig. 13. In the calculations for this plot $I_0(A)$ was made equal to $I_0(B)$ and the absorptivity of the chromophor at wave length A was made equal to ten times the absorptivity at wave length B. In general then, negative deviations such as this will occur whenever the light beam is composed of two or more monochromatic rays possessing different absorptivities.

In all previous discussion it has been assumed that ideal monochromatic light has been used. In practice this is never quite realized, even with the best monochromators. For that type of monochromator where the entrance and exit slits are equal in width the distribution of light energy among the rays emerging from the exit slit has the shape indicated in Fig. 14. The data in that figure were obtained by allowing light from a mercury arc to fall on the entrant slit of a Beckman D.U. spectrophotometer. With the wave length dial set at 546 mμ, the slits could be closed to a very small value and still pass sufficient energy to achieve a balance of 100 on the transmittance scale. On either side of this position the intensity of the 546 mμ line fell off rapidly. Not until the wave length dial was set to below 532 or above 558 mμ was the intensity of the 546 line reduced to zero. Thus a monochromatic ray will be passed to varying degrees over a whole range of dial settings and will have its peak value when the dial setting corresponds to its wave length (assuming the monochromator is properly calibrated).

On the other hand, when the entrant slit is illuminated with white light it follows that the band interval from 532 to 558 mμ will also be passed. The intensity of individual wave lengths will vary relatively as indicated in Fig. 14. Since the 546 mμ ray is the most intense, it will make the greatest

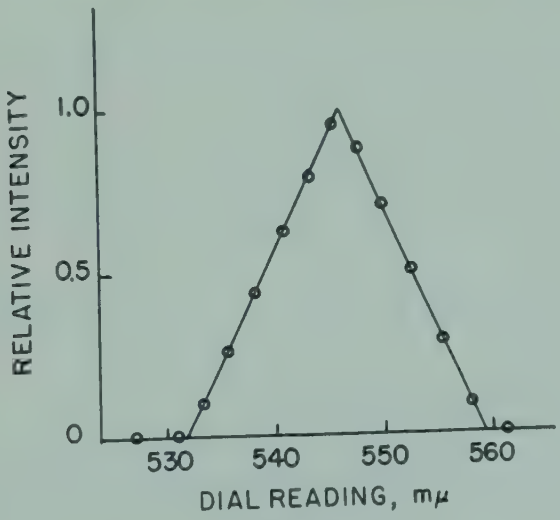

FIG. 14

contribution to the apparent absorbance while the rays on either side will make correspondingly lesser contributions. Outside the interval limits there will be no contribution except as there is white light in the system. A more extended treatment of this band width problem has been given by Gibson (1949).

From these considerations it is apparent why deviations from the absorption often occur. Conformity to the law will occur only when the absorption spectrum has a relatively flat peak, so that the absorptivities of all the rays passed by the exit slit will be equal. In such a case all of the rays are attenuated equally. Furthermore, whenever absorbance measurements are made on a side of an absorption band apparent absorption law deviations must always occur. Indeed such apparent deviations must occur if the Bouger-Beer relationship is valid. Negative absorption law deviations of the sort discussed here seldom cause the analyst more than a little extra effort in making his calibration curve. Later it will be shown that negative deviations of this sort cause a slight decrease in the precision of analytical measurements.

When an absolute absorptivity measurement is to be made it becomes necessary to make a correction for the slit width effect. Eberhardt (1950) has shown that such corrections may be made empirically by measuring the absorbance as a function of the width. As the slit width is narrowed the gain of the amplifier must be increased. Where the slit width error is important, it will be found that the apparent absorbance increases as the slit width is decreased. After a sufficient number of such measurements has been made, the absorbance can be plotted as a function of the slit width interval. This plot is then extrapolated to a zero slit width and the absorptivity is computed from that extrapolated value.

Certain precautions must be observed in this procedure. For example, as the slit width is narrowed the amount of energy passed to the photo tube is proportionately decreased. Consequently, the signal-to-noise ratio of the amplification system will diminish with a resulting decrease in the precision of measurement. Thus the absorptivities at the narrowest slit width and at the highest resolution are in general the least precise. This will, therefore, introduce a larger uncertainty in the points closest to the zero slit width reading.

These considerations also show the need to indicate the slit width whenever absolute values of the absorptivity are reported.

b. Deviations Due to Chemical Effects

There are a wide variety of factors which affect the absorption spectrum of a solution or play a role in the chemical reaction which produces the absorbing species. Such factors often make it appear that the absorption law is failing, where instead it is the absorbing molecule which is being altered in some fashion.

The most common variables to be considered are the following: pH, redox effects, electrolyte concentration, colloid content, temperature, impurities and gross alterations in the solvent composition. In addition to these there is the further factor that as the concentration of the absorbing species is increased, it may itself alter the properties of the medium and thus produce changes in the spectrum. These several factors will now be discussed briefly.

(1) pH and redox effects. A great many absorbing species possess acid or basic groups or both. Consequently, as the pH of the solution is varied the concentrations of its conjugate forms will vary. Since these forms usually have different spectra it is evident that the absorbance will change with pH.

The acid-base reaction is commonly formulated as $A \rightleftharpoons B + H^+$ where A, B and H^+ represent the activities of the acid, base and hydrogen ion species. In colorimetric measurements, it is not activities which are measured but rather the concentrations of the colored species and therefore suitable conditions must be chosen or else corrections must be made. The absorbance at a particular wave length of a solution containing A and B in equilibrium may be expressed as $\log I_0/I = A = a_A bC(H^+/H^+ + K_a) + a_B bC(K_a/H^+ + K_a)$. In this expression C equals the sum of the concentrations of A and B and is of course independent of pH. $(H^+/H^+ + K_a)$ and $(K_a/H^+ + K_a)$ are terms indicating the fractions of C which are respectively in the A or B forms. K_a is the acid ionization constant of the indicator being used. The sum of the two fractional terms is unity. When, therefore, $H^+ \gg K_a$ the concentration of the A form will approach C while the concentration of the base form will approach zero. The reverse is true when $K_a \gg H^+$. When $H^+ \equiv K_a$, i.e., when the $pH = pK_a$ of the indicator, the two fractional terms will be equal and the concentration of A will equal that of B.

At any particular wave length, the absorptivities a_A and a_B will have particular values. As the wave length is varied these absorptivity terms will of course vary. If there is no overlap of the spectra of these two species then in the region where A absorbs, a_B will be zero and the left hand term disappears from the absorbance equation, with the resulting simplification of the expression. On the other hand, if there is overlap of the spectra then it follows that at one (but sometimes more than one) wave length a_A will equal a_B. At such a point the absorbance is pH independent. Regardless of whether the dye is in the acid or base forms the sum of the two fractional absorbance terms will always equal the same value. Such a point is termed an isosbestic point.

It is illustrated in Fig. 27 in the 460 mμ region. Here are given the absorption spectra of solutions containing either one or the other of the conjugate species A and BQ and two different mixtures of them A′ and A″. Since the concentrations of A and BQ were equal to each other and to the sum of the concentrations in A′ and A″ the isosbestic point appears. Some authors recommend making determinations of dye concentrations at such points, particularly when the pH cannot or is not to be controlled.

Apparent deviations from the absorption law may appear in acid-base systems. When the solution is poorly buffered, the addition of increasing quantities of one of the absorbing species may produce a progressive shift of the pH with the result that the absorbance will show either a negative or a positive deviation with increasing concentration. By working at a pH several units removed from the pK of the species this deviation can be avoided when successive ionization stages are well separated. Otherwise, it is necessary to buffer adequately.

A great many organic materials are rather easily oxidized by atmospheric oxygen. Such oxidations are usually favored in alkaline media. When such cases are encountered it will be observed that the spectra vary with time and with concentration. The calibration curves obtained under these conditions may be quite irregular even though reproducible under a fixed set of conditions.

Methylene blue is one example of this type of effect while the phosphomolybdite blue produced in the Follen and Wu method for the determination of glucose is another.

(*2*) *The effect of electrolyte concentration.* It has been known for many years that the salt concentration has an effect on the absorption spectra of many species. The effects which are observed are usually of small magnitude and are caused by inter-ionic attraction or ionic-dipole interaction between the absorber and the electrolyte. In Fig. 15 a remarkable set of data, originally published by Stearns (1950) is presented. It shows the spectral shift produced by adding as little as 1 % sodium chloride to a solution of Stillbene Yellow 2G. Had a calibration curve been prepared from a dye standard contaminated with salt, a spectral variation with increasing concentration would result, causing an apparent deviation.

(*3*) *The effects of colloids.* Colloid substances when present in solution along with a dye often exert a very pronounced effect upon the color. This is due to the fact that the dye molecule is absorbed at the surface of the colloidal particles as a result of van der Waal and coulombic forces. When this occurs profound alterations in the electronic and vibrational states of the molecule result. Sörenson (1909) noted such effects with proteins. He demonstrated that pronounced color changes occurred in solutions of various indicators when protein was added to the solution. This in spite of the fact that the indicator solution was buffered against pH changes.

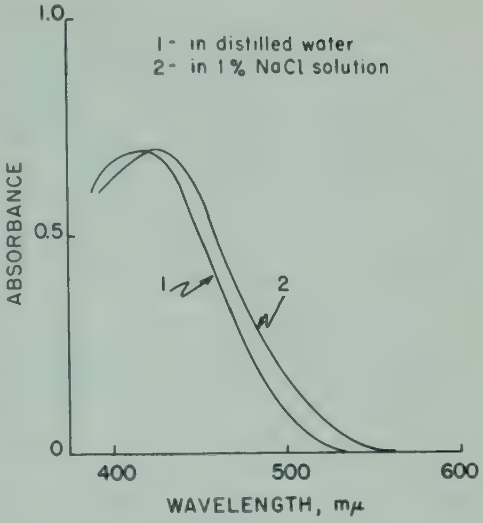

FIG. 15

Detergents present in solution in moderate concentrations form colloidal aggregates known as micelles. The micelle will consist of many molecules of detergent clumped together to form an oily drop of colloidal dimensions. The surface of the micelle is charged when an ionic detergent is used. Such micellar bodies have a very strong attraction for a great many of the more complex dye molecules; pronounced color changes may be induced by merely adding detergents to solutions of such molecules.

While there is a very extensive literature on this subject in which many complicated interactions are revealed between colored substances and detergent micelles, it will only be necessary to show the interaction between a quaternary ammonium salt and a simple dye, methyl orange, to illustrate how extraordinary the effects may be. In Fig. 16 data are presented for methyl orange in a buffered solution at the pH 1 to which various amounts of octadecyl-trimethyl ammonium chloride are added. It is apparent that a very striking interaction occurs here with a profound spectral alteration (Hiskey and Downey, 1954).

Carroll and Thomas (1949) have shown similar pronounced color changes when methyl orange was adsorbed on colloidal aluminum hydroxide. These few examples illustrate the importance of investigating the effects of any colloidal impurities which may be present.

(*4*) *Temperature effects.* It is well known that the equilibria of many chemical reactions are effected by temperature change. When one of the products of such an equilibrium is being measured spectrophotometrically, care must be taken to exclude fluctuations of temperature. A dramatic illustration of this effect may be had by taking a dilute solution of phenolphthalein and buffering it with ammonium chloride and ammonium gas. If the concentration of the ammonia is so adjusted that the solution is colorless or only slightly tinged when at 80°C or so, it will be found to develop a strong pink color on cooling to room temperature and lower. This effect is quite reversible and is due to the change of the auto-ionization constants of the solvent. If the solution were buffered at very low or very high pH values this effect could not be demonstrated. Only when neutralization of the dye is approximately 50% complete, i.e. where pH = pK, will the effect of temperature be sizeable and of the order of 3% per degree centigrade. By

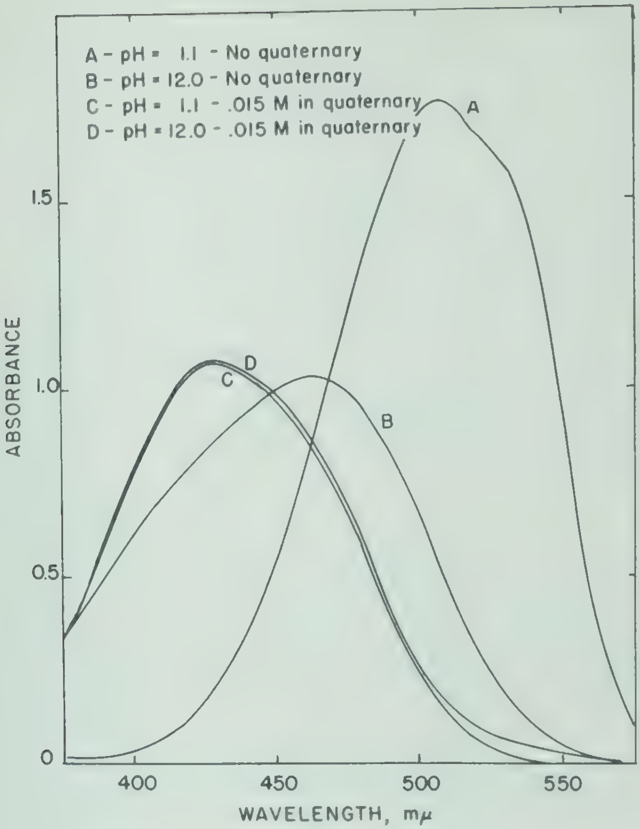

FIG. 16

working at pH values several units larger or smaller than the pK of the dye the effects of temperature may be minimized.

Not always, however, is the effect of temperature reversible. In a great many instances heating of a solution may result in thermal decomposition of a colored substance or it may result in a hydrolytic reaction which permanently alters the absorption spectrum of the dye. For example, diamine violet N has been shown by Holmes (1923) to give a characteristic absorption spectrum when dissolved in water. It is stable over a period of 24 hours or more, providing the temperature is kept below 25°. If the solution is heated to 85° the spectrum is altered and does not return to its original form when cooled back to room temperature.

(5) *The effect of concentration.* A great many cases are known where increasing the concentration of an absorbing species has a pronounced effect upon the color of the solution. Usually these color changes are associated with a change in the composition of the absorbing species in solution. For example, if cobaltous (II) chloride is added in successively increasing amounts to a 4 *M* HCl solution, it will be observed that the solution changes

from a pink color in the dilute region to take on a purplish tinge as the concentration is increased. In the very highest concentrations, the solution will be a typical cobalt blue. This is due to a shift in the equilibrium between the pink $(Co(H_2O)_4^{++}$ ion and the blue $CoCl_4^{=}$ ion, as a result of increasing concentration. Equilibrium effects of this sort have been extensively reported for the higher molecular weight dyes.

In a great many instances mixtures of colored substances do not behave as would be predicted from the Bouger-Beer relation. This is usually due to interaction among the absorbing species. In the analysis of complex mixtures it is therefore necessary to establish the additivity of absorbances. The assumption of additivity may never be made without at least some preliminary testing.

Another cause for apparent deviations from the absorption law has been suggested by attempts to determine the concentration of various substances in cell structures. This deviation is related to alterations in the absorption spectrum caused by the state of aggregation. Insofar as the absorbing molecules are randomly oriented within the cell structure no deviation from the absorption law occurs.

Commoner and Lipkin (1949) have examined the relationship between the absorbance for unpolarized light and the number of molecules of a type possessing such a low symmetry that at least one optical axis different from all possible other axes exists. They have derived these relationships for three degrees of orientation of the given optical axis relative to the light beam. They have shown that when there is complete random orientation of the axis then the absorbance is equal to $\alpha N/3$. In this expression N represents the number of absorbing molecules, and alpha equals the absorbtivity per molecule when the optical axis of the molecule and the electric vector of the light beam are parallel. That the absorbance is only $\frac{1}{3}$ of αN arises from the fact that there are three possible axes of orientation of the molecule when randomly oriented.

In a second case which they treated they assumed that all the optical axes of the molecules were to lie in a stack of planes with each plane perpendicular to the light beam but with the axis of the individual molecules in each plane randomly oriented. Under these conditions it can be shown that the absorbance is equal to $\alpha N/2$. Thus the apparent absorbance is 50% larger for a given number of molecules in this case. Since the molecules are here restricted to only two degrees of randomness the coefficient becomes $\frac{1}{2}$ instead of $\frac{1}{3}$ as above. In both of these cases, however, it is apparent that the absorption law applies without deviation.

If the optical axes of all the molecules are parallel to each other and the light beam is in a plane perpendicular to these axes then the absorbance relation becomes Eq. 15. Examining this relationship reveals that when N

is allowed

$$A = \log \frac{2}{10^{-\alpha N} + 1} \tag{15}$$

to become extremely large the absorbance becomes equal to 0.3. Only at very small values of αN will there be an apparently linear relationship between A and N. This linear relationship between A and the number of molecules will exist to a value of somewhat less than 0.15. Above this value the slope of the curve changes until it approaches zero, and A approaches 0.3 as a limiting value. In other words, the amount of light absorbed in this region becomes independent of the number of molecules. It is seen, therefore, that if a completely oriented aggregate of optically anisotropic molecules such as we are considering here is examined with monochromatic polarized light, then the amount of incident light absorbed can never be above 50%. This same type of behavior is exhibited by a sheet of polaroid.

Commoner and Lipkin have asserted that there is considerable cytological evidence that some degree of orientation exists in practically all cytological structures other than the liquid vacuoles. Furthermore, most biologically important molecules are optically anisotropic. Hence the assumption that the concentration of such an absorbing substance in a cell structure can be calculated from the ratio of its absorbance to the absorbance of a given number of molecules of the substance in solution is not valid.

This point of view has been disputed by Pollister and Swift (1950) who argue that such orientations are not as frequent as Commoner and Lipkin assert and cite many experimental cases where the anticipated deviation did not appear. Thorell and Ruch (1951) have presented data indicating that even with nucleic acid the effect appears to be negligible in tissue. Additional discussion may be found in papers by Wilkins (1950) and others. It seems likely that this form of apparent deviation is not too common in biological tissue.

(6) *Effects of solvents.* The absorption spectrum of a compound is dependent to some extent on the solvent medium. If the composition of a solvent is grossly altered, such as by the addition of alcohol to water or a shift from water to some non-aqueous solvent, it may be expected that the spectrum will not only alter in the position of the absorption maximum but that it may be profoundly altered as regards its absorptivity and the shape of the entire spectral curve.

(7) *Irreversible changes in spectra.* Irradiation with light, x-rays, or alpha or beta rays often cause irreversible changes in the chemical structure of a colored compound. Such changes lead to a permanent alteration in the absorption spectrum. In the same way intense illumination may have a

bleaching effect on a great many substances. Indeed, certain compounds are so extremely labile that they will be bleached by the light shining on them in some of the commercial colorimeters.

Another irreversible effect which is occasionally noticed is that of the absorption of dyes onto the glass surfaces of the absorption cell. This kind of process has an unusual effect on the accuracy of the data. When such a dye solution is first placed in the absorption cell, adsorption on the cell faces occurs. Subsequent samples of the same dye analyzed in the same cell will cause little or no further alteration in the quantity of dye adsorbed at the water-glass interfaces, but the effect of this adsorption is to increase the apparent absorbance of all subsequent solutions by a fixed amount.

4. Precision of Spectrophotometric Measurements

Precision is a measure of the reproducibility of determining a quantity. Accuracy on the other hand expresses the correctness of a measurement, i.e., the degree to which a measurement corresponds to the truth. In spectrophotometry, the principal concern is with precision. This arises from the fact that one cannot, *a priori*, compute the absorptivity of a particular molecule. The customary method of work, therefore, is to make suitable dilutions of a known standard solution and then measure the absorbances. Analysis for this particular substance is then made by determining the absorbance of an unknown solution and comparing the measured value with the calibration curve previously derived.

The precision of a spectrophotometric analysis is determined by two general types of factors, instrumental and chemical. Among the instrumental factors the sensitivity and time stability of the instrument are the most important. Sensitivity may be defined as the ability of the instrument to discriminate small differences in transmittances. The greater this sensitivity the smaller will be the absorbance difference needed to produce a detectable variation in the reading of the instrument. If is obvious that the sensitivity is limited by any fluctuations of the electrical circuit and of the light intensity during the course of the measurements. The time stability of the instrument is associated with the constancy of the zero and 100% transmittance settings during the course of a series of measurements. Both of these instrumental limitations will be reflected in the transmittance uncertainty term which shall here be represented as $\Delta(I/I_0)$.

The chemical and manipulative factors that influence the precision of a spectrophotometric determination are as follows: There is a first requirement that the reaction used in producing the absorbing molecular species must be stoichiometric in character if data of the highest precision is to be achieved. Where a colorimetric reaction is subject to side reactions, temperature effects, effects of atmospheric oxidation, fading on exposure to the beam of light, and the like, precise results cannot be obtained, no matter how good the spectrophotometer used. Where the colorimetric reaction is stoichiometric in character then the limitation of the precision is often imposed by the technique of the analytical operator. Here it will be important to remember that the weight of the sample taken and the final volume to which this unknown sample is brought must also be precisely known. If such measurements are made with a precision of 1 % or less then the final results obtained from the spectrophotometer can be no better than this. Less frequent but by no means unimportant sources of error in spectrophotometry may arise from a lack of reproducibility in preparing the optical faces of the cuvettes. It was seen above that surface films affect the reflectances of these optical surfaces. Care must be exercised to thoroughly degrease and cleanse the optical cuvettes and maintain them in that state during the course of any analysis. A further factor to bear in mind is the need to minimize the heating effects. With an increase in temperature the density of solutions generally diminishes thereby producing an apparent dilution of the sample. Except for some organic solvents these effects usually produce errors of only parts per thousand per degree centigrade.

All the uncertainties in both the instrumental part of a spectrophotometric method as well as the chemical and technique part will be ultimately reflected by an uncertainty in the determination. This uncertainty may be evaluated by making a number of measurements of the same unknown. For the overall uncertainty these individual determinations should include every step in the entire process. Where the instrumental uncertainty only is to be evaluated, repeated analyses of a standard solution is all that is required.

From a series of n determinations an average value for the concentration may be found by taking the arithmetical mean of the n values. The individual values will differ from this median by an amount which will vary for each measurement. The average of these n differences (without regard for sign) may also be taken to get an average deviation. This average deviation from the mean reflects the overall uncertainty. The relative error will be given by $\Delta C/C$, i.e., the average deviation divided by the mean. An evaluation of this quantity for the instrumental error only will demonstrate that there is an optimal concentration at which the analyst should work.

a. Optimal Concentration

For any spectrophotometer there is an optimal concentration at which the relative error will be minimized. At all other values of the concentration the relative error is substantially larger. Two opposing factors are responsible for this situation. The first is that a unit change of transmittance represents a different concentration difference in the various parts of the transmittance scale. In solutions of high transmittance a unit change of transmittance represents a very small change in concentration. Conversely, in the vicinity of zero this same unit of transmittance corresponds to a very great concentration change. This suggests that one should work in the most dilute solutions in order to obtain the greatest sensitivity. However, in making a concentration measurement it is the difference between 100 % and the actual value of the transmittance that is determined. The larger this difference is the bigger the concentration term and therefore the smaller will be the relative error for any given concentration uncertainties. These two opposing factors, therefore, operate to produce a region of minimum relative error.

The relative error, $\Delta C/C$, may be evaluated in the following way:

$$-abc = -A = \log I/I_0\,. \tag{16}$$

Evaluating dC first,

$$-\,dC = -(1/ab)dA = \frac{0.43}{ab}\cdot\frac{dI/I_0}{I/I_0}\,. \tag{17}$$

Since

$$-\,C = \frac{1}{ab}\log I/I_0\,. \tag{18}$$

Therefore

$$\frac{dC}{C} = \frac{0.43\,dI/I_0}{I/I_0\log I/I_0}\,. \tag{19}$$

The value of the relative error function may be approximated by substituting finite values for the infinitesimals as in Eq. 20.

$$\frac{\Delta C}{C} = \frac{0.43\,\Delta I/I_0}{I/I_0\log I/I_0} \tag{20}$$

$\Delta(I/I_0)$ is the uncertainty of reading the exact position on the transmittance scale. In most commercial instruments its value will be of the order of 0.01 to 0.002 of the total scale and it is a constant quantity when the instrument is functioning normally. It may be evaluated experimentally by taking a solution and measuring its transmittance repeatedly. Each measurement should include the repositioning the cuvette and setting the zero and 100 % position of the scale. $\Delta(I/I_0)$ may be taken as twice the value of the average deviation.

A plot of the reciprocal of $I/I_0 \log I/I_0$ as a function of the transmittance of the solution is given in Fig. 17. $\Delta I/I_0$ is left unspecified. Here it can be seen that the relative error is infinite for 0.0 and 100 % transmittances. At the intermediate transmittances the relative error decreases to a minimum and then rises again. In the region between 20 and 65% the curve is nearly flat and hence a minimum and nearly constant error is achieved by working in this region. By differentiating the error function with respect to I/I_0 and setting that differential function equal to zero we can obtain the point of minimum error. This can be demonstrated to correspond to a transmittance of about 36.8% or an absorbance value of 0.43. These considerations therefore impose certain concentration limitations on the analyst.

The optimum range for analytical work derived above has been evaluated on the assumption that there are no apparent deviations from the Bouger-Beer relationship. Ringbom (1939) has developed a method which allows an exact evaluation of the range where the precision is at a maximum regardless of apparent absorption law deviations. He proposed that photometric data be plotted with per cent transmittance as the ordinate against the logarithm of the concentration as the abcissa. If a sufficient concentration range has been investigated, such a curve always passes through an inflection, as shown in Fig. 18. The precision is always greatest when $\Delta I/I_0$ divided by $\Delta C/C$ is at a maximum. This can be found from the plot in the figure by looking for the inflection of the curve, that is where the curve has its deepest slope. If the system has no apparent deviation from the Bouger-Beer law, the general form of the curve is always the same, with the inflection occurring at 36.8% transmittance. If the system does not appear to follow the Bouger-Beer relation, the inflection will occur at different values of the transmittance and these may be evaluated by inspecting the curve.

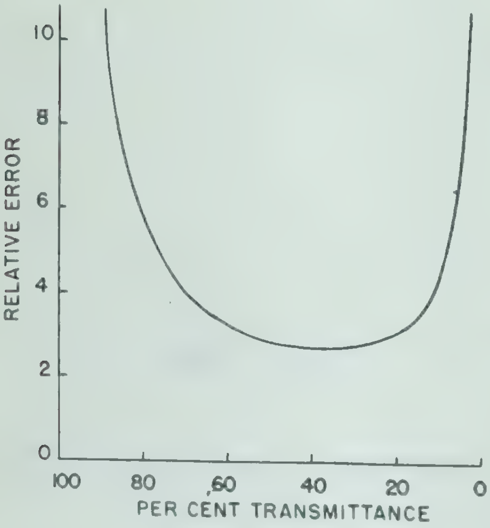

FIG. 17

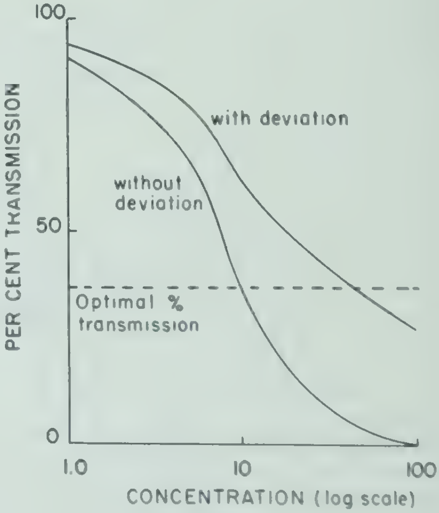

FIG. 18

b. Optimum Precision

The question now to be considered is what is the maximum precision which may be obtained with any instrument. Equation 20 shows that this will be equal to that precision which is obtained when the transmittance is 36.8 % (for the ideal case). Under these conditions the relative error is equal to $2.72 \times \Delta I/I_0$. In other words, if the uncertainty of reading the transmittance scale is equal to 1 %, then the uncertainty of the determination from instrumental factors alone will be 2.72 %. Consequently there will be no need to improve the precision of the other operations to better than 2.72 %. In order to get more precise analyses, it is necessary to improve the instrument so that the uncertainty of reading the transmittance scale is reduced substantially. In some of the better spectrophotometers this has been done to such an extent that the uncertainty of reading the transmittance may be as little as 0.1 %. With such instruments the relative error of measurement may be reduced to as little as one part in 300. Improving the sensitivity of the instrument would of course reduce the error still further.

There is another way to achieve a great enhancement of precision. This is by the use of the relative transmittance measurement, (Hiskey 1949). The reason why the relative transmittance method is much more precise than the absolute types of measurements arises from the fact that it determines the difference in absorbance between the reference standard and the unknown. Therefore, if there is a certain error for the determination of this small difference, but the total absorbance of the sample is extremely large, then this small error is divided by the large total absorbance resulting in a very much smaller relative error.

To demonstrate what this error function looks like in a number of different cases, i.e., where the absorbance of the reference standard is set at different values, a generalized plot of the error function is presented in Fig. 19.

The curves presented are for those cases where the reference absorbance has values of 0.0, 0.109, 0.22, 0.43, and 1.74. Here it can be seen that when the absorbance of the reference standard is zero, an error curve identical with that in Fig. 17 is obtained. As the absorbance of the reference standard is progressively increased, the relative error falls quite rapidly in the vicinity of 100 % relative transmittance. Were it not for the rapid decrease in light transmittance with increasing absorbance, the error could be reduced at will. In practice it is impractical to work with an absorbance in excess of about 1.74, because the transmittance of the reference standard is only 1.8 %. With this limitation recognized, however, a considerable gain in precision is still achieved.

Examination of the plot shows that when the absorbance equals zero the minimum error is equal to 2.72. This occurs when the absorbance of the unknown is 0.43 or has a transmittance of 36.8 %. In the case where *A* has a value of 1.74, the minimum error is 0.25 or about $\frac{1}{11}$ that obtained with the solvent as the comparison standard. Thus, wherever it is possible to apply a relative transmittance method, a tenfold gain in precision may be anticipated over what one might normally expect out of this particular instrument. Greater care is necessary in the preparation of the samples in order to achieve this increased precision; the treatment presented here applies only to those cases where the chemical system and technique have the requisite precision. There is also the underlying assumption in this case that the absorption law holds. Where an absorption law deviation exists this will affect the precision. In general, if the deviation is positive enhanced precision will be obtained whereas the reverse will be true if the deviation is negative in character. Hiskey and Young (1951) have treated this problem in detail.

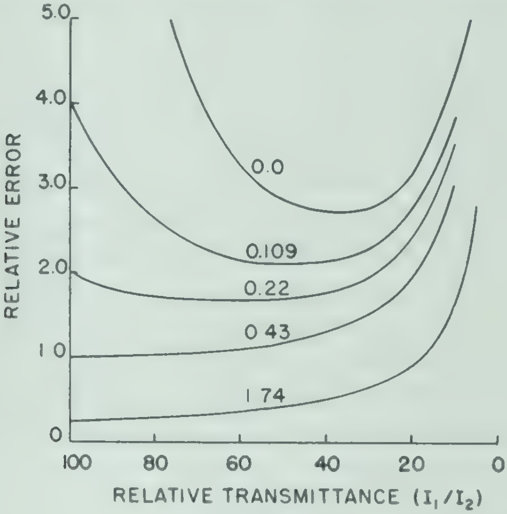

FIG. 19

Having thus briefly outlined some of the more important principles of spectrophotometry, it will now be useful to us to consider the many different ways that these principles have been applied.

III. Applications

1. ANALYSIS OF ONE COMPONENT SYSTEMS

If one has a sample containing but one absorbing substance, then a measurement of its absorbance at one suitable wave length will suffice to determine its concentration. If it is known that the substance obeys the absorption law, then the concentration is simply proportional to the measured absorbance.

For maximum precision of analysis the concentration of the absorber is so chosen that the transmittance is within the 20–60% region as discussed above. On the other hand, if the absorption law is not obeyed, then, in general, it will be advisable to make a Ringbom plot and choose from this plot the concentration range in which to work. It will also be necessary, when the absorption law is not followed, to have such an empirical calibration curve for the concentration-absorption relationship.

With respect to the choice of wave length to be used for any particular determination, maximum sensitivity and precision will generally be obtained by operating at the wave length corresponding to the absorption maximum. If the absorption maximum is relatively broad compared to the band width passed by the slit then little error may be expected as a result of the inability of the operator to set the wave length scale of the instrument reproducibly.

Another factor affecting the choice of wave length is the sensitivity of the photoreceptor at that wave length. In those regions where the photocell is most sensitive in its response, the slit width needed to balance the instrument at 100% transmittance will be least. Where the photoreceptor response is poor, the slit width will be correspondingly larger. It has been shown above that the larger the slit width the greater is the wave length interval passed by the instrument and therefore the greater is the likelihood for deviations from the absorption law to occur. Where a particular absorbing substance may have two absorption bands, one in a region of good photoreceptor response while the other one in a region of very poor receptor response, consideration should be given to this factor. It may be found quite desirable to work with an absorption peak of lesser absorptivity merely because the photoreceptor sensitivity is greater and therefore the slit width is very much narrower.

The sensitivity of a determination may be improved considerably by increasing the length of the transmittance cell. Where provision is made in the instrument for path lengths in the soltion of 2, 5, 10, or 50 cm long, it can be anticipated that the concentration of the solution may be reduced to ½, ⅕, ⅒ and $\frac{1}{50}$ that of the solutions in the 1-cm cell, without altering the absorbance. When the very long absorption cells are employed it is important to take precautions to eliminate dust from solution and in general to watch for scattering effects due to these and other colloidal matter.

2. Problems of Multi-Component Assay

a. Simple Two-Component Case

When two components are present in solution with absorption spectra which do not overlap, the concentration of each may be determined either by a relative or by an absolute measurement just as though they were separately in individual solutions. If their absorption spectra overlap, then

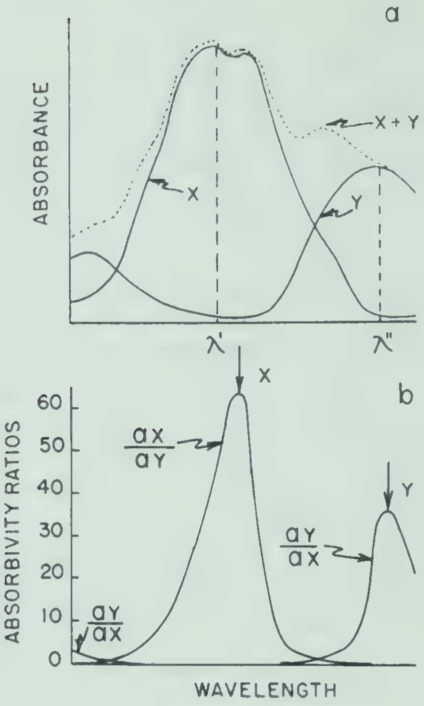

FIG. 20

compensation for this overlap must be made in the course of their simultaneous spectrophotometric determination.

An illustration of such a situation is represented in Fig. 20a. The two components, x and y, have absorption maxima at wave lengths λ' and λ'', respectively. The dotted line represents the light absorption characteristics of the mixture; the two heavy lines give similar information for the two components if measured separately.

At any particular wave length it is assumed that the absorbance of the mixture will be the sum of the two individual absorbances so that for λ' and λ'' Eqs. 21 and 22 apply. To obtain the values for the concentrations of x and y the two equations are solved simultaneously as follows: (23) (24) (25).

$$A' = A_x' + A_y' = a_x'c_x + a_y'c_y\,; \tag{21}$$

$$A'' = A_x'' + A_y'' = a_x''c_x + a_y''c_y\,. \tag{22}$$

To obtain the values for the concentrations of x and y the two equations are solved simultaneously as follows:

$$c_y = A''/a_y'' - a_x''/a_y''c_x\,. \tag{23}$$

Substituting Eq. 23 into 21 there is obtained

$$A' = a_y'/a_y''A'' = a_x'c_x - a_y'/a_y''a_x''c_x\,. \tag{24}$$

On rearranging we have

$$c_x = \frac{A' - A''(a_y'/a_y'')}{a_x' - a_x''(a_y'/a_y'')} = K_x(A' - k_xA'') \tag{25}$$

Since the term $\frac{1}{a_x' - a_x''(a_y'/a_y'')}$ is a constant, it may be designated as K_x. Similarly, a_y'/a_y'' may be represented as k_x. This reduces the apparent complexity of the expression, leaving only two constants, k_x and K_x to be evaluated. A similar expression can be derived for c_y as in Eq. 26. Note, however, that the constants K and k are different in the two expressions.

$$c_y = \frac{A'' - A'(a_x''/a_x')}{a_y'' - a_y'(a_x''/a_x')} = K_y(A'' - k_yA') \tag{26}$$

Examination of these two equations reveals that they are quite general for any two-component case. For example, if the two absorption bands had no overlap then this would mean that in Eq. 25 a_y' would equal zero, because component y would have no absorption at wave length λ'. This would mean that k_x would equal zero and therefore the term k_xA'' would also equal zero. It would also mean that in the denominator of that expression the term $a_y'a_x''/a_y''$ would also equal zero and therefore disappear from the denominator. Under these conditions then Eq. 25 would reduce to $c_x = A'/a_x'$ or in other words to the simple one-component case. A similar result may be shown for c_y by incorporating the assumption that x does not absorb at λ'' in Eq. 26.

The procedure for determining the concentration of x and y in the presence of each other, assuming that the absorption law holds, is as follows: First, the absorption spectrum of components x and y are measured independently over the wave length interval of interest. From such data it is possible to evaluate the constants K and k for any two values of the wave length. Now by taking two measurements of the absorbance of a mixture of components x and y at the two wave lengths thus chosen, it becomes possible to solve Eqs. 25 and 26 for the concentration of these two constituents.

The question immediately arises as to the method of choosing the two wave lengths for the two absorbance measurements. It has been seen previously that for a single component it was best to work at a wave length where the absorptivity is greatest providing other factors such as steepness of the peak, photoreceptor sensitivity, etc., do not make this region unsuitable. In the two-component case the wave lengths are chosen at those points where the ratio of the absorptivities, i.e., a_x'/a_y' and a_y''/a_x'' are at a maximum and yet in a wave length region where the absorptivities of both components are appreciable. Reference to Fig. 20b shows where these wave lengths are for the case under consideration. It will be seen that a_x'/a_y'

peaks at a wave length to the right of λ'. This is the better wave length to use in determining component x in this mixture. Similarly a_y''/a_x'' peaks somewhat to the right of λ'' and this wave length should also be used when analyzing the mixture.

Recently Kuratani (1951) has shown the usefulness of plotting the ratio A'/A'' as a function of the concentration of either of the components. When this is applied to mixtures of pure substance and in particular to those cases where there is no interaction among the two components, then this ratio is a simple straight line function of the percent concentration of either component. The ordinate will have a value equal to a_y'/a_y'' when the concentration of x is zero. When the concentration of x is 100% then the value of the ratio will equal a_x''/a_x''.

The advantages of this function for chemical analysis are that; a) the ratio is independent of cell thickness, b) no solvent is needed, c) it is unnecessary to weigh out the sample and finally, d) where no deviation occurs the calibration curve can be obtained by calculation if the absorption spectra of the pure substances have been determined. This method of approach is particularly applicable in the infrared region and has been tested by Kuratani on mixtures of the cresols, of the chlor-octanes and of the chlor-octanes with octyl alcohol. Where there is interaction among the two components resulting in an alteration of their absorption spectra, then it becomes necessary to make a complete calibration curve over the region of interest to the analyst.

b. Two Components with Absorption Law Deviations

Corrections for deviations from the absorption law are more difficult in a two-component than in a single-component determination. With two components there are two factors which may cause deviation from the absorption law. One factor is that a certain substance may not of itself follow the absorption law when it is alone in solution; it can be expected that this same failure will occur when it is present in mixture. On the other hand, certain pairs of absorbing substances may, when present in solution together, interact with each other with such weak bonding that easily dissociable aggregates occur, but which nevertheless have a profound effect on the absorption spectra of both.

The first of these factors may be corrected for as follows: Prior to any analytical work, a plot of the absorbance-concentration relationship is made for each of the components. This is illustrated for component x by the solid line in Fig. 21. The absorptivity a_x', which is used in subsequent calculations (Eq. 25) is derived from this curve by taking some arbitrary value of the absorbance, A_0'', and dividing it by the corresponding concentration c_x^0. This procedure assumes a straight line relationship to exist as shown by the dashed curve in the figure. This is the source of the error in calculating c_x by means of Eq. (25). c_x will be in error to the extent that the absorptivity is wrong.

To correct for this situation the calculated value of c_x is marked on the abcissa. Then a vertical line is extended from c_x upward until it meets the dashed line. At that point, a horizontal is drawn until it intercepts the true absorbance curve. By dropping a perpendicular to the abcissa from that intercept the true concentration of x is found. Where both substances show such deviations the process must be repeated on a similar curve for y. The answers derived by this correction will approximate the true concentrations quite closely and this represents one of the simplest corrections that can be made for deviation situations of this sort.

When deviations are due to a chemical interaction between two components in solution then a somewhat more complicated method of correction is required. If x and y comprise the entire sample a plot of either absorbance A' or A'' is made as a function of the per cent of x in the sample. This must be done for a fixed weight of sample and that weight is a parameter in all subsequent analyses for this pair of substances. The resulting curve or curves will show the extent to which deviation exists with this mixture. Consequently with such a calibration curve one can make a corrected analysis merely by measuring either A' or A'' and referring to the appropriate curve. Using A' or A'' in conjunction with the two calibration curves will serve as an additional check on the analysis.

If the sample containing the mixture has one or more additional constituents which do not otherwise interfere with the determination, it then

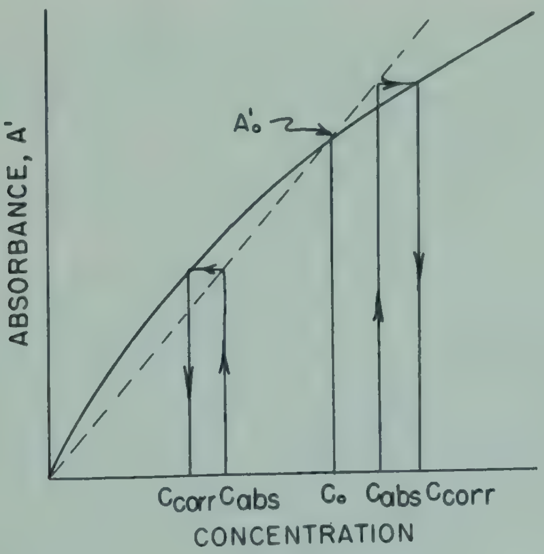

FIG. 21

becomes necessary to prepare a range of these absorbance-concentration curves for different percentages of these diluting substances.

Suppose, for example, that 50% of the sample were inert constituents while the remaining 50% were distributed between components x and y. A plot of A' and A'' as a function of the per cent of x in that part of the sample reserved for x and y, i.e. $100\left(\frac{x}{x+y}\right)$, will give a curve which is similar to the preceding one except displaced to a region of lower absorbances. When a series of such curves has been prepared in which the per cent of the diluent has been adjusted over the probable range in the samples to be analyzed then one has sufficient data to make corrected analyses. When the measurements for these calibration curves are actually made, one uses a weight of sample, in the case where 50% of the material is inert, that is exactly twice that where no inert material is present, etc. In this way constancy of precision is maintained. The A' and A'' values are of course halved when the plot is made.

After this calibration chart has been prepared the unit sample weight or some known multiple thereof is taken for analysis. A' and A'' are measured, normalized by dividing by the weight multiple and then compared with the calibration charts on the λ' and λ'' values. By a process of trial and error there will be found one composition for which the observed values of A' and A'' agree with the calibration curves or with some interpolated points. When those points have been found the analyst will simultaneously know the concentration of the diluent in the system as well as the relative concentration of x and y. He will also have made, in principle, an exact correction for the deviation.

In practice, it is not always necessary to run very many of the calibration curves for A' and A''. This is particularly true when the dilution caused by the inert constituents does not affect the degree of interaction between x and y. In such cases curves measured at the extreme concentration ranges of the inert constituents will suffice. The curves for intermediate dilutions may then be interpolated with a considerable saving of effort.

c. *Relative Method for Determining One Component in a Mixture of Two*

A simple and useful method for the determination of one constituent in a mixture of two has been developed by Allan and Hammacher (1952). Application of this method presupposes that only one of the constituents is of interest while the other is to be ignored. When this is so, a reference standard is prepared from the constituent which is to be ignored.

Let us assume that this constituent is termed y and that it has its absorption maximum at wave length λ''. The constituent of interest however will be termed x and it will have its absorption maximum at λ'. Now the absorbance of this mixture may be expressed as in Eq. 21 and 22. In this method however, comparison is not made against a solvent but only against a solution of component y. One of the features of this method is that the concentration of y need not be known.

It is necessary to choose a concentration for y such that in the vicinity of wave length λ'' the apparent absorbance measured is very nearly zero, i.e., $A'' = a_y''(c_y)_s$. The subscript s here refers to the reference solution. If this reference standard is now compared against the unknown solution an absorbance difference measurement will be obtained.

The absorbance difference equations for the two wave lengths are given as:

$$A' = a_x'c_x + a_y'c_y - a_y'(c_y)_s ; \tag{27}$$

$$A'' = a_x''c_x + a_y''c_y - a_y''(c_y)_s . \tag{28}$$

If these two equations are solved simultaneously in order to get c_x it will be found that the resulting equation is identical in its outward form to Eq. 25, the terms involving $(c_y)_s$ cancelling out. However A' and A'' do not refer to absolute values of the absorbance of the solution but only to differences in absorbance between the unknown and the reference solution.

It will be recalled from Eq. 25 that k_x is equal to the ratio a_y'/a_y''. If this ratio is approximately known from an examination of the absorbance spectrum of component y then k_x will be approximately known. It is not a requirement that k_x be known very precisely since A'' is generally going to be made rather close to zero, and therefore an error in k_x will have little effect on the precision of the entire result. K_x, on the other hand, can be considered to be a calibration constant which needs to be determined with solutions of known composition. Where the normal absorption law holds K_x will be constant throughout the range of interest. If this is not the case a table must be constructed showing the dependence of K_x on the solution being analyzed.

In practice a decision is first made as to the two wave lengths to be used for the analysis. These wave lengths are chosen in accordance with usual criteria for two-component systems. Then a solution of compound y is prepared to be used as reference and of such a concentration that it will always nearly equal the concentration of y resulting during analysis. The size of the sample should be of such a value that the relative transmittance is within the 60 to 20% range. Because the use of a reference solution will result in less light being transmitted to the photo tube than if pure solvent were employed, it will be necessary to widen the slit in order to balance the instrument at 100% transmittance.

Analysis is made of an unknown sample by comparing the absorbance of its solution with that of the reference standard at wave length λ''. The absorbance difference will usually be close to zero but it may be either positive or negative. If the absorbance is negative, meaning that the transmittance of the reference standard is less than that of the unknown, the analyst proceeds to make the unknown a temporary reference standard while measuring the absorbance difference between it and the reference standard. Next the wave length is adjusted to λ'. Here it will be found that the absorbance difference is always positive. With these two measurements and utilizing constants K_x and k_x the concentration of component x may be evaluated. Where there are gross deviations from the absorption law it will be necessary to refer to a calibration curve relating concentration of component x to the absorbance observed when the solution contains an equal amount of component y.

Application of this method to situations such as this will in general result in a precision which is somewhat better than that obtained when using the absolute spectrophotometric method for the two-component case. It is not however as good as using two reference standard solutions of both components x and y. A consideration of this problem has been made by Hiskey and Firestone (1952). They have shown that if the absorptivity ratios $a_x/'a_y'$ and a_y''/a_x'' are determined in a relative way then a precision of one part per thousand may be anticipated in suitable analytical reactions applied to two-component assay. For those desiring such precision a study of the reference cited is recommended.

d. Absorbance Difference Spectra

Heretofore when relative absorbance measurements have been considered such measurements have been restricted to a single wave length. With the development of recording spectrophotometers it is now convenient to make such relative absorbance measurements over the entire wave length region covered by the instrument. The relative spectrum so obtained represents the absorbance difference curve between the reference standard and the sample being compared to it. If the composition of the reference solution and the solution to be analyzed are the same the absorbance difference will be zero at all wave lengths. Thus whenever a relative absorbance curve is a straight line with a value of zero it may be inferred that the standard and the unknown solution are exactly matched in composition. Our concern therefore will be with the direct location of or the estimation of the conditions necessary for such matching of spectra.

To illustrate this problem consider first the three absorption spectra presented in Fig. 22. Curves A and B represent the spectra of solutions containing specified concentrations of component A and B respectively. Curve C is the spectrum obtained when a single solution possesses both components in identical concentrations with those in A and B.

In Fig. 23, spectra are given when solution *A* is successively compared with solvent and reference solutions of *A* with 0.5, 0.9, 1.0, 1.1, 1.5 and 2.0 times the concentration of the solution under study. It can be seen that as the concentration of the reference standard increases, the absorbance difference spectra steadily diminishes in height and when the concentrations are exactly matched a straight line with zero absorbance is obtained across wave length interval. Increasing the concentration of the reference standard further produces negative absorbance.

A point of considerable practical interest is that in the vicinity of the absorption peak there is a change in curvature as the concentration in the reference standard passes through the match point. By compressing the wave length scale this change of curvature can be sharpened considerably.

To illustrate these absorbance difference spectra where more than one constituent is present the data plotted in Fig. 24 should be examined. Here solution *C*, containing both components *A* and *B* in admixture is compared against a reference standard of component *A*. Again it is seen that as the concentration of *A* increases in the reference standard, there is a reduction in the absorbance difference. Finally, when the concentration of *A* in the reference standard and solution *C* are identical the absorbance difference spectra which remains is exactly equal to that of component *B*. A further increase in the concentration of component *A* in the reference standard now produces a negative absorbance spectra.

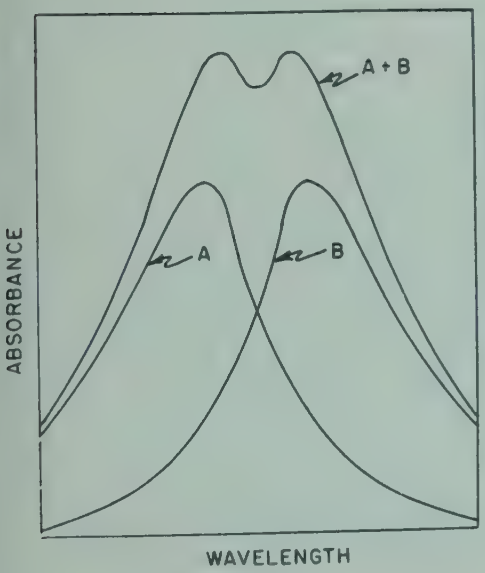

FIG. 22

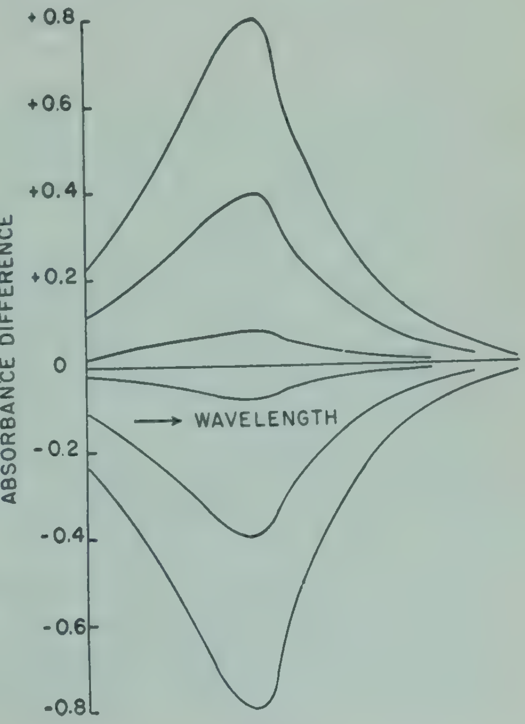

FIG. 23

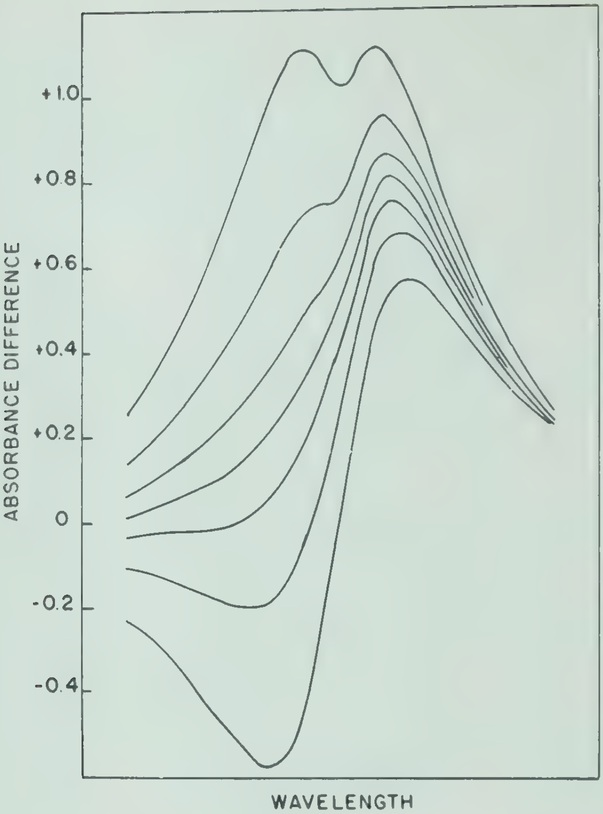

FIG. 24

By the use of reference standards containing both components *A* and *B* it would be possible by a tedious process of hunting to find those combinations of concentrations which would exactly match the absorption spectrum of the unknown solution.

(*1*) *Variable reference technique.* The difficulties of such an approach to the analysis of a multi-component system of this sort have been largely overcome by the use of a variable reference technique devised by Jones, Clark, and Harrow (1951). In their procedure a titration vessel, the reference cuvette, and a suitable pumping system are so connected that when the pump is activated the contents of the titration vessel are circulated continuously through the reference cuvette. Burettes containing standard solutions of the components whose concentration it is desired to determine are set in such a way that titration may be made directly into the titration vessel. Starting then with a known volume of solvent in the system the concentration of any component may be adjusted to any desired value merely by adding an appropriate volume of one of the standard solutions. To determine the concentration of a single species in solution, when present by itself, requires a simple titration with the standard solution of the constituent being determined. When a straight line is achieved through the wave length interval being examined addition of the reagent is stopped. If one overshoots the "end point" a known quantity of solvent is added to the vessel to bring it back to the proper side. After which addition of the standard reagent is continued.

The titration of a multi-component system is scarcely more difficult. A certain amount of experience with this technique is desirable in estimating when one constituent is very nearly compensated for. Apart from that, however, the procedure is the same. After scanning the spectrum of the mixture additions of the standard reagent of the preponderent constituent is begun. This is continued until that peak is substantially reduced. It will be observed that the absorption peaks of the other components then become more pronounced. Now each of these in turn may be reduced until finally a straight line is again achieved. It is necessary of course to keep a record of the quantities of standard reagents which are added as well as the total volume of solvent introduced into the system at the time that the match point is achieved. The details of the application of this method to the analysis of a number of pharmaceutical products and dye substances may be found by consulting the original reference.

In essence this technique converts the recording spectrophotometer into a null-indicating device for determining an absorptiometric end point. Admittedly the spectrophotometer is a relatively complicated and expensive device to be used in this way. Nevertheless there are some very obvious advantages to this technique which will make it one of increasing usefulness as more laboratories acquire such recording spectrophotometers.

A most important field of application for this particular technique is in the analysis of mixtures of components, particularly where three or more components are involved. It has been previously shown what the complications were in analyzing a system of only two components. With more than two components the complexities of solving the systems of equations make these determinations extremely tedious and difficult unless special computing machinery is available. However with the variable reference technique the spectrophotometer is not only a spectrophotometer but it eliminates the computing machine as well as the difficulties of the computation.

Another very great advantage to this technique is that there are no difficulties introduced as a consequence of deviations from the absorption law due to interactions among the several components. Inasmuch as all of the constituents in the reference vessel will be present in identical concentrations as in the unknown sample, any interaction among them will be exactly duplicated. Since the absorbance of the two solutions is identical at all wave lengths, when the matchpoint is reached, instrumental errors are largely compensated for. This technique will only fail in those systems where light absorption characteristics alter as a result of irreversible changes during the course of the analysis.

One weakness of the variable reference technique is the relative slowness with which spectral data can be obtained from most recording instruments. However, here too some progress has been made. Recently, Daly (1951) described a spectro-comparator which presents absorbance difference data on a cathode ray screen. The whole spectral region is scanned about ten times per second while absorbance difference measurements are made about 1500 times per second. Thus an instantaneous presentation of absorption difference spectrum is effected. The instrument described by Daly is presently being marketed by Unicam Instrument Lted., Cambridge, England. In the United States, the American Optical Co., is marketing a comparable device.

e. *Analysis in the Presence of Background*

Up to now the discussion has been confined to cases in which the identities of all the components in the mixture under investigation are known. It has however been pointed out that unless the unknown and the final reference solution have the same composition it is not likely that the absorbance difference could be reduced to zero at all wave lengths. If it is impossible to obtain zero absorbance at all wave lengths by addition of the standard employed then it is obvious that the unknown must contain some material not present in the standard solution. This situation is often met with in the analysis of natural products, such as vitamin A in various fish oils or the determination of aureomycin in the fermentation of liquor. In these instances the solutions have a residual color or background color due to other constituents than the one of interest to the analyst. In such instances even if all of the important constituents could be removed there would still be a residual spectrum remaining in the system. One of the more complicating situations here is that in natural products the character of this residual or background spectrum will vary from sample to sample. Where conditions of manufacture are standardized it often becomes a fairly reproducible component but in the majority of cases this is not so.

(*1*) *Absorbance difference technique.* There are three principle ways of attacking this kind of problem. The first method consists of finding some chemical reaction which will destroy or so alter the absorbing species as to shift its spectrum to a quite different region of the wave length scale. If such a reaction can be made quantitative then it is evident that the only portion of the absorption spectrum remaining will be due to the background color. As an example of this one may cite the destruction of vitamin A in cod liver oil by means of ultraviolet radiation. On the other hand, any acid-base or redox reaction which will destroy or so alter the absorbing species that it is quantitatively removed will be just as suitable. By comparing the sample containing the constituents to be analyzed along with the background against the sample whose constituent has been effectively removed, an absorbance difference spectrum is obtained which is characteristic of the absorber in question. With the aid of a specimen of the pure compound it becomes a simple matter to construct a calibration curve for this analysis.

(*2*) *Variable reference.* A second approach to this problem is by use of the variable reference technique described above. For this purpose a specimen of the pure substance must be available so that it may be added dropwise to the titration vessel.

As the concentration of the reference standard is increased the absorbance of the desired constituent will be subtracted from the composite spectrum of the background and the component of interest. If the concentration in the reference standard is made larger than that of the unknown solution then an absorption minima will appear where formerly a maxima had existed. The point of equal concentrations will be recognized as that point where the curvature in this vicinity changes sign. This had been pointed out previously in the figures above. By looking at a whole set of such spectra one can then interpolate and arrive at a result in rather good agreement with the truth.

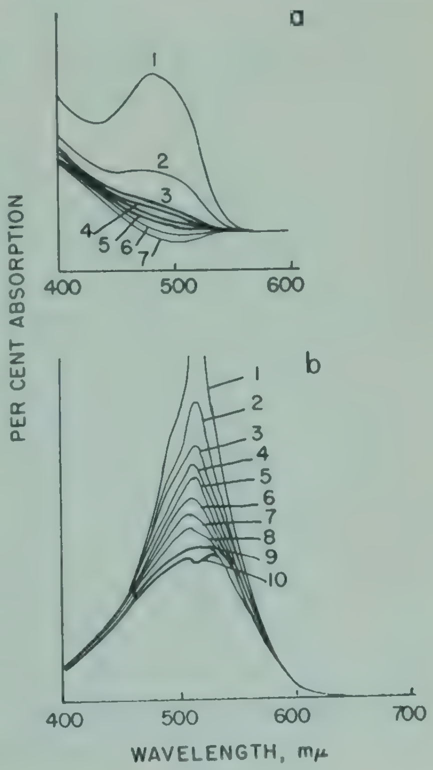

FIG. 25

In Figure 25 two illustrations of this technique, copied from the paper of Jones *et al.*, 1951, are presented. Figure 25a and Table II show the spectrophotometric titration of a mixture containing F.D. and C Yellow No. 6 and caramel. Given a series of seven curves the problem is to select the most reasonable background. There is obviously still an excess of dye in the solution cell for curve No. 2 because there is some absorption at the wave length where the dye absorbs strongly. Curve 7 represents the case where a deficiency of dye is present in the solution cell because there is an unnatural deficiency in the absorption in the region where the dye absorbs. With a little experience, curve 5 may

TABLE II

Determination of F.D. and C Yellow No. 6 in the Presence of a Caramel Background

Curve	Yellow No. 6 mg.		Volume
1	0.0		1000
2	6.02		
3	8.00		
4	8.50		
5	9.00		
6	9.50		1010
7	10.00		
	Estimated Comp.	8.9 mg/l	
	Actual comp.	9.0 mg/l	

be chosen as the most reasonable curve representing background. Had no correction been made for the background the single component analysis for Yellow No. 6 would have given a result of 10.35 mg/l. If one assumes a linear background absorption in the region from 420 to 580 mμ and makes appropriate corrections therefore, analysis would then give a result of 8.5 mg/l of dye present. But by the variable reference technique a result of 8.9 mg/l is obtained. This compared with the known value of 9.0 mg/l. Hence in this case the variable reference technique gives the most accurate results. Figure 25b shows the curves obtained in another determination. The dye in this case was F.D. and C Red No. 21 which has a sharp peak at 517 mμ with a slight shoulder at 480–490 mμ. Examination of the different curves indicated that the most likely background curve is number 8, corresponding to a concentration of 4.9 mg/l. The actual concentration in this instance was 5.0 mg/l. In curve 9, a dip is already apparent in the curve, and in No. 10 this dip is quite pronounced. Thus it is seen that the point where an over titration occurs can be easily distinguished. The backround substance in this instance was not given but it is evidently a material possessing an absorption peak in the same general vicinity as Red No. 21.

(3) *Base line technique.* A third and somewhat older method for handling the background problem is the method developed by Morton and Stubs (1946). Their method, called the base line technique, assumes a linear absorption of the background in the vicinity of the absorption peak of the constituent to be determined. Except for this approximation, the method is otherwise exact. In their hands it has been a very powerful tool in the

analysis of vitamin A in various fish oils. For a detailed description reference should be made to the literature cited. However, the essentials of their approach are relatively easily grasped and will be presented here.

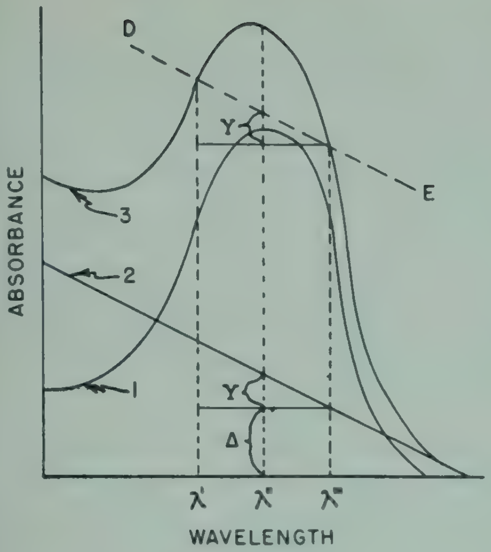

FIG. 26

In Fig. 26 there is diagrammed a situation which is amenable to their method of attack. Curve 1 represents the absorption curve of the pure component with a peak at λ''. At λ' and λ''' the absorptivities are equal but less than that at λ''. The background assumed in their solution is the straight line 2. The composite curve obtained when both were present together in solution is given as curve 3. It can be seen that the total absorbance at wave length λ'' on curve 3 is given as the sum of the absorbances of the pure component plus the background absorbance at that wave length. In order to determine the magnitude of the absorbance due to the backround it is necessary to know two things: First the slope of the background curve and second the extent to which this curve is displaced upward along the ordinate.

The slope may be found quite easily by measuring the absorbance of the composite solution at wave lengths λ' and λ'''. When background is present these will not be equal. Consequently, a line DE drawn through the composite absorbances at these two wave lengths will give us that slope. It is to be noted that *DE* is parallel to the background line 2 in the figure. Now by simple triangulation it is possible to find the displacement at λ'' due to this slope. That displacement is given in the figure by the letter Y.

The total displacement of the absorption peak due to the background material is given as the sum of Y and Δ at λ''. The determination of Δ may be accomplished in the following fashion: Remembering that for the pure substance the absorptivity at λ'' is greater than at λ''' so that

$$A_1'' = k(A_1'''). \tag{29}$$

The constant k is determined from the spectrum of the pure substance and is seen to be less than unity. The A_1'' and A_1''' are absorbance terms due component 1. In the composite curves A'' will always be greater than A_1'' by an amount equal to the sum of Δ and Y. Therefore

$$A_1'' = A'' - \Delta - Y.. \tag{30}$$

On the other hand A''' will differ from A_1''' by an amount equal to Δ, i.e.

$$A_1''' = A''' - \Delta. \tag{31}$$

Substituting Eqs. 30 and 31 into 29 gives

$$A'' - \Delta - Y = k(A''' - \Delta). \tag{34}$$

Here it is seen that of the five terms A'', A''' and k are susceptible to direct measurement. Y has been determined previously and therefore Δ is the only unknown in this equation. Consequently, the correction may be now effected.

This base line technique has been applied by Morton and colleagues to a number of different analytical situations. It will be very useful for the analyst to study these applications. The advantage of the base line technique is its applicability to situations where background is evident and where the analyst does not have available a recording spectrophotometer. Where the background curve deviates from linearity it is not as precise a method as the variable reference technique but with the instrument limitations mentioned above it may be the only one available to the analyst.

f. Derivation of Two Completely Unknown Spectra When Pure Components Are Not Available

Quite often the situation arises where two or more components are present in solution with the spectra of one or more unknown to the analyst. It had been shown previously that by use of the variable reference technique resolution of the unknown spectra might be affected. Suppose for example that there are present in solution only two constituents and that pure solutions of one of these components is available for use as a reference standard. In such a case, by determining the absorbance difference spectra as a function of the quantity of the standard solution added to the variable reference cell it is possible intuitively to locate that situation where the standard component has been completely compensated. The residual absorbance difference spectrum then represents that of the unknown constituent. Such mixtures are relatively easy therefore to resolve and the unknown spectrum may be quickly deduced.

However, the case to be treated here is somewhat more complicated. It is assumed that there is available no pure standard solution of either of the constituents. At first thought it might seem that the resolution of such a situation is indeed hopeless. However, by a mathematical device described by Hardy and Young (1948) such a situation may be resolved providing that some means is available for altering the relative distribution of the two unknown components in the solution. To illustrate this technique consider the spectral curve given in Fig. 27. The solid line used in representing the spectral curve A' and A'' are for two solutions containing different proportions of the same two constituents. In the case being discussed the solutions here represented were those of methyl orange 2×10^{-5} molar buffered at a pH of 1.0 to which variable quantities of a quaternary ammonium salt had been added. The problem now is to deduce from these two curves the spectra of the two components. To do this one proceeds in the following fashion:

The absorbance A' for the first solution is given at any wave length by reference to the curve. This absorbance is equal to any wave length to

$$A' = a_A c_A' + a_{BQ} c_{BQ}' . \tag{33}$$

The terms a_A and a_{BQ} in this expression represent the unknown absorptivities of component A and BQ at any particular wave length. The unknown concentrations of component A and BQ are represented by c' to indicate that for this particular solution they have a specific value. Now, by adding more of the long-chain quaternary ammonium salt the spectrum may be altered to that given by A'' for which Eq. 34 applies.

$$A'' = a_A c_A'' + a_{BQ} c_{BQ}'' \tag{34}$$

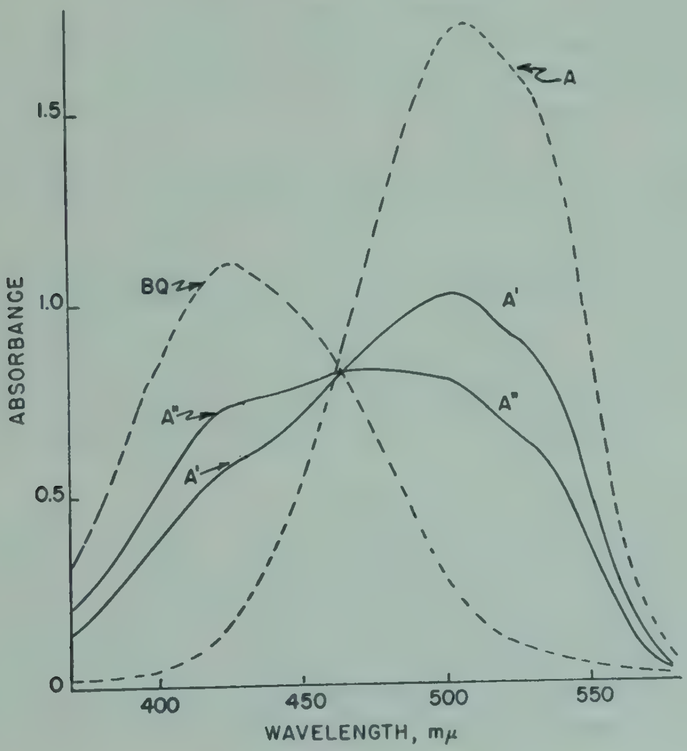

FIG. 27

Again as in Eq. 33 the absorptivities of the pure components at any given wave lengths are the same despite the fact that they will obviously vary with wave length. The relative amounts of A and BQ in this solution will differ from that in the first solution. It can be seen that these two equations contain four unknown concentrations in addition to the two unknown absorptivity functions. The complexity of this situation has led to the impression that such a case as this could never have a unique solution. However, by defining a new function A_D and operating with this function, resolution of the two spectra becomes possible. In Eq. 35 A_D is arbitrarily defined as shown.

$$A_D = A'' - kA' \tag{35}$$

In this expression k is also an arbitrary constant. When Eqs. 33 and 34 are substituted into it there results Eq. 36.

$$A_D = a_A(c_A'' - kc_A') + a_{BQ}(c_{BQ}'' - kc_{BQ}') \tag{36}$$

Inspection of the equation reveals the following interesting facts. If a value for the arbitrary constant k can be so chosen that c_{BQ}'' equals kc_{BQ}' then the value of the second term of the right hand part of this equation is zero for all wave lengths and the A_D function now is proportional to the absorptivity of component A alone. Similarly, if a different value of k is so selected that c_A'' is made equal to kc_A' then the first term of the right hand side of the equaion becomes zero for all wave lengths and the function is now proportional to the absorptivity of BQ. It should be evident therefore that a qualitative determination of the shape of the absorption spectra of these two components can be effected providing appropriate values for k can be selected.

Returning now to the two absorption spectra, A' and A'', it is seen that as the quaternary ammonium concentration was increased the absorbance in the 510 mμ region fell. This suggests the disappearance of a species with an absorption peak in that vicinity. In the 420 mμ region the effect of added quaternary salt was to raise the absorbance, suggesting therefore an increase in concentration of a species having an absorption peak in this vicinity.

To isolate the spectrum which has its peak in the 510 mμ region it is necessary to make $(c_{BQ}'' - kc_{BQ}')$ equal to zero. Since c_{BQ}'' is larger than c_{BQ}' the values of k chosen should be greater than unity in this particular instant. If arbitrary values of k such as 1, 1.2, 1.3, 1.5, etc., are chosen and a plot of the function A_D as given in Eq. 35 is made, there is obtained a family of curves which resolve an absorption peak in the 507 mμ region more sharply as k is increased. If k is made larger than is necessary to bring the term ($c_{BQ}'' - kc_{BQ}'$) to zero then a negative absorbance function will appear in the 410–420 mμ region. Thus by looking at a whole family of these curves each with a difference value of k it is possible to pick intuitively a curve which approximates the correct value of k. For this intuitively chosen value of k the calculated points of the A_D function are plotted in Fig. 27 and are compared with the known absorbance curve for the acid form of methyl orange. The known curve is given by the dashed line and is seen to be in quite close agreement with the points calculated. It must, of course, be understood that after obtaining the A_D function, the peak value of that function was normalized with known data of the spectrum in order to make the two curves coincide at the peak.

By repeating this process using values less than unity and again intuitively choosing the appropriate value of k which appeared to give a single spectrum peaking in the vicinity of 426 mμ the data for the BQ spectrum were obtained. The calculated points are shown by the crosses and the dashed line again refers to the known spectra of the pure component. These data were also normalized against the known absorption spectrum of BQ. It is thus seen that quite satisfactory qualitative resolution of the two unknown spectra may be achieved by this approach. Had the individual spectra of the pure components not been available then it would have been necessary to discover the normalization factor in order to know the absolute absorptivities at any wave length. This cannot always be achieved however. On the other hand, if the problem is one involving an equilibrium mixture such as in the case here then it is possible to calculate that factor.

For an equilibrium mixture of two components the sum of the concentrations of the two forms is always a constant and therefore Eq. 37.

$$c_A' = \text{const.} - c_{BQ}'; \qquad c_A'' = \text{const.} - c_{BQ}'' \tag{37}$$

The A_D function will equal

$$A_D = a_A(c_A'' - kc_A') = a_A \cdot \text{const.}\ (1 - k) \tag{38}$$

when c_{BQ}'' is equal to kc_{BQ}'. Therefore

$$a_A = \frac{A_D}{\text{const.}(1 - k)}. \tag{39}$$

Thus it can be seen that the absorbance of component A at any wave length is equal to the value of A_D at that wave length divided by the product of the constant and $(1 - k)$. If the amount of the unknown mixture can be known in terms of moles per liter then the absolute molar absorptivity may be calculated with Eq. 39. On the other hand, where the sum of the weights are only known then if the molecular weights of the two species differ markedly expressing the concentration in weights per liter will give results for the absorptivity which will be somewhat in error.

The application of this method may be made not only to systems consisting of two substances at equilibrium but it may also be applied to situations like the background problem previously discussed. In such an instance where neither the background spectrum nor the spectrum of the component of interest is known this procedure may be used providing a method is found for altering the concentration of either the background material or the substance of interest. This approach may be also extended to the analysis of a three-component system. When that is done, however, three equations of the type given in Eqs. 33 and 34 are required with an additional term in each. Unfortunately the analysis of such multi-component systems becomes very complex and is quite laborious.

g. Determination of Absorption Spectra in Opaque Substances

Recently a method has been devised for the measurement of the optical constants of solid and liquid substances by means of reflection measurements in the infrared. The method is applied to substances so highly absorbing that the preparation of samples for measurement by transmission is not feasible. Its application is restricted mostly to the solid state but it also may be applied to liquids which exhibit very high values of the extinction coefficient in their absorption band. This reflection method yields not only the absorption coefficient but also the refractive index of the substance for the wave length measured. Only a brief description of the essential details of this method will be given here. The interested reader is referred to the very illuminating paper by Simon (1951).

The fraction of light transmitted by a solid absorbing medium is usually expressed by that variant of the absorption law given in Eq. 40.

$$I/I_0 = e^{-\alpha 1} = e^{-(4\pi n\kappa 1)/\lambda} \tag{40}$$

In this expression α is the absorption coefficient and is equal to the product of the absorptivity and the concentration when applied to the cases considered previously. The thickness of the absorbing layer is here given as 1. Textbooks on fundamental optics demonstrate that α is equal to $\frac{4\pi n\kappa}{\lambda}$ when n is equal to the refractive index and κ is termed the extinction coefficient. λ is the wave length of radiation in vacua. In a dispersive absorbing medium the refractive index is complex and is written as

$$n = n(1 - i)\kappa. \tag{41}$$

Here n is the real part of the index while i is the square root of -1. If appropriate substitution of these terms is made into Snell's law and then into the Fresnel relation previously listed extremely complex equations result relating the fraction of light reflected at a given angle of incidence in terms of n and κ. Since n and κ represent two unknowns it is necessary to make reflectance measurements at two discrete angles for any particular wave length so that two equations may be solved simultaneously to get both n and κ. Direct solution of these two equations turns out to be impractical but by computing tables of these functions for angles of incidence equal to 20 and 70 degrees respectively as a function of n and κ it is possible to make a relatively rapid graphical solution of the problem. It is necessary of course to introduce an appropriate correction for polarization of light due to reflection off the various prism surfaces in the spectrophotometer employed. A simple method for achieving this correction has been devised.

In application then, a beam of monochromatic light either polarized or unpolarized is allowed to fall on the surface whose optical constants are to be determined and the intensity of the reflected ray is measured for the two discrete angles listed above. By reference to the two graphs a single set of values of n and κ will be found which satisfies the reflectance relationship. The true reflectance of a surface must however be corrected for by multiplying the measured reflectance by an appropriate correction factor. Although only recently devised, this method has proved its usefulness not only in studies of glasses but in a wide variety of studies of high polymers and of organic crystals as well as many liquids. In its present form this reflection method is much less sensitive than the transmission method but it appears capable of further refinement.

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Chapter 4

ULTRAVIOLET ABSORPTION SPECTROPHOTOMETRY

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I. Introduction

Spectroscopic methods often offer the most convenient, sensitive, and accurate means of obtaining information about the chemical constituents of biological systems. Indeed there are instances in which there is no other practicable alternative to spectroscopy for obtaining the desired information. These methods have been widely used for qualitative and quantitative analysis in biochemistry, and for the elucidation of structure of hormones, nucleic acids, proteins, coenzymes, vitamins and other compounds of biochemical interest. The recent combination of microscopy with spectroscopy has afforded a new powerful tool for the cytochemist.

The spectroscopic method depends on the interaction of radiation with matter and the subsequent measurement of the intensity or energy of the radiation as a function of wavelength or frequency. One may observe the emission or absorption of radiation, scattering of radiation with a change of wave length, photochemical and photoelectric effects.

The absorption of radiation has been the most widely employed of the phenomena mentioned above. Indeed the application of the techniques of absorption spectroscopy have become so widespread as to be commonplace in the chemical laboratory and in industrial control mechanisms. Ready availability of instruments for absorption spectroscopy has in many instances led to the application of the method without adequate understanding of the nature of the absorption process, of factors which influence the process and of the influence of the spectroscopic instrument itself on the data obtained.

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This chapter will deal with the absorption of radiation in the range of the spectrum bounded by the transmission limit of air (*ca.* 1900 A; Ladenburg, *et al.*, 1932) on the short wave length end, and by the nominal beginning of the visible spectrum (4000 A) on the long wave length end (see Fig. 2, p. 77). The material to be presented is divided into four sections. First, there is a qualitative discussion of theories concerning the nature of the absorption process. The second section deals with the correlation of molecular structure and ultraviolet absorption and with environmental factors which influence absorption. Third, commercially available instruments for absorption spectroscopy will be described and the effect of instrumental variables on the results obtained will be discussed. Finally, application of absorption spectroscopy to biological problems will be illustrated by an annotated guide to the literature and a listing of sources for data on absorption spectra of pure compounds.

II. Theory

It will be helpful in later discussions if the reader has in mind certain ideas that have contributed to the development of spectroscopic theory. It is probably easiest in a short section such as this to begin with certain assumptions regarding the light emitted by atoms and progress to ideas of atomic and molecular structure and the relation of these ideas to light absorption. For a more extensive narrative account of the development of these ideas see Hoffman (1947).

The light emitted by atoms that have been excited in any one of a number of ways is restricted to certain very narrow bands of radiation referred to generally as emission lines. These may be few or many and their wave length is characteristic for the emitting atom. These facts may be accounted for by assuming (1) that the emission of radiant energy by an atom represents the transition of the atom from a state of higher energy to one of lower energy and the converse for the absorption of light, (2) that the magnitude of the energy transition is proportional to the frequency of the emitted or absorbed radiation and (3) that an atom may exist only in one of a series of separate energy states (omitting the kinetic energy of translation) which are characteristic of the structure of the atom.

The first assumption recognizes the conservation of energy.

The second assumption follows from the quantum theory of radiation of Planck and from Einstein's photoelectric equation derived from Planck's theory. This assumption can be expressed as:

$$\nu = \frac{E_i - E_f}{h} = \frac{\Delta E}{h}$$

where ν is the frequency of the radiation emitted or absorbed, h is the quantum constant, and E is the change in energy of the atom in passing from the initial energy state E_i to the final energy state E_f. These energy states represent different configurations of the electrons surrounding the nucleus of the atom. Changes in energy of this sort are hence called electronic transitions.

The third assumption is simply a statement of the conditions necessary, when taken together with the first two assumptions, to yield the emission or absorption of light as the narrow bands characteristic of the atom instead of any or all frequencies independent of the atomic structure. Niels Bohr developed and applied this idea to a model of the hydrogen atom and obtained good agreement between the frequencies he was able to calculate and those observed. He was also able to define in physical terms the Rydberg constant, which had been previously arrived at empirically from study of the hydrogen spectrum.

Bohr's approach was refined and extended to more complicated spectra by Sommerfeld and later by others. Their work led to the development of wave mechanics and quantum mechanics.

When the absorption spectra of molecules were examined it became apparent that the observed absorption could not satisfactorily be explained on the basis of atomic electronic transitions alone. It became necessary to develop ideas of molecular electronic transitions and of quantized transitions other than electronic. These will be discussed in order.

The absorption of ultraviolet and visible light is the result of a molecular electronic transition involving primarily the valence electrons. Current ideas concerning the electronic structure of the molecule may best be summarized by reference to Fig. 1. In Fig. 1A the compounds ethane and ethylene are illustrated. The dots about the symbols represent the valence electrons; the inner shell electrons are not shown. These are the so-called "Lewis dot structures," named for G. N. Lewis who first used them to illustrate his ideas of the chemical bond (Lewis, 1916). The bond between the atoms is represented by the sharing of electrons. The shared electrons are considered to belong to either atom of the bound pair. The single bond constitutes the sharing of two electrons, the double bond the sharing of four electrons and the triple bond the sharing of six electrons. These dot structures have been quite useful in organic chemistry but they do not correctly represent the situation as it is now thought to exist.

In Fig. 1B the same structures are represented by a pictorial approximation of the quantum mechanical structure. In this representation cognizance is taken of the fact that electrons cannot, as suggested by the dot structure picture, properly be located exactly, but rather must be represented by probability functions or charge density diagrams (Pauling, 1945; Coulson, 1952). In this structure the single bond is again composed of a shared pair of electrons which are restricted to a volume located between the two atomic nuclei. The volume containing the bonding electrons is symmetrical about a line joining the bonded atoms. For the purposes of molecular spectroscopy these electrons have been designated *sigma* electrons and are represented by the lower case Greek letter σ. Sigma electron bonds also exist between atoms bonded by double or triple bonds. As illustrated for the case of ethylene, the extra pair of electrons participating in the double bond do not lie between the bonded atoms as suggested by the dot structures. These extra electrons, known as *pi* (π) electrons, occupy sausage-shaped volumes symmetrically disposed about a plane that passes through the bonded atoms. In the case of the triple bond the third pair of electrons (one pair of σ electrons and two pair of π electrons) is restricted to volumes of similar shape to those occupied by the π electrons of the double bond. Each pair of π electrons is symmetrically disposed about a plane which intersects the other plane at right angles along a line joining the bonded atoms. In larger molecules containing extensive conjugated unsaturation it is thought that adjacent π electron "sausages" may fuse so that electrons of the system may be free to move over the entire conjugated portion of the molecule. *Pi* electrons of double or triple bonds that are not part of a conjugated system remain restricted to volumes such as those illustrated for the case of ethylene.

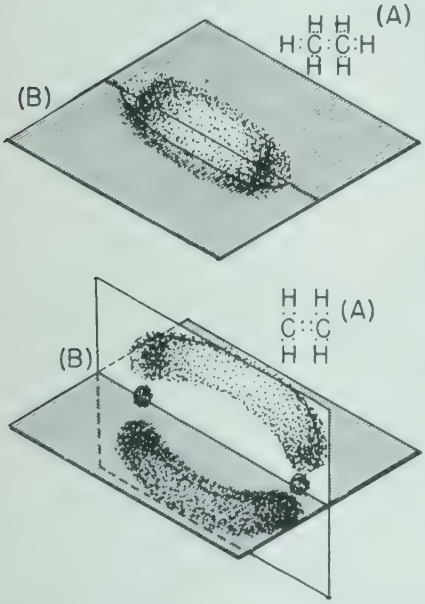

FIG. 1. Schematic representation of the electron distribution in the carbon-carbon bond in ethane (above) and ethylene (below). The carbon-hydrogen bonds have been omitted for clarity.

With the assumptions given above for the atomic spectra interpreted in terms of the molecular electronic structure it is possible to account for the general position of an absorption band due to an electronic transition involving the valence electrons.

However, the details of observed molecular spectra cannot be accounted for on the basis of electronic spectra alone. It is necessary to assume that the natural vibrations and rotations of the atoms within the molecules are also quantized. From this it can be said that the change in energy of a molecule during absorption (or emission) of light can be written and the sum

$$\Delta E_{\text{total}} = \Delta E_{\text{electronic}} + \Delta E_{\text{vibrational}} + \Delta E_{\text{rotational}} .$$

This last statement is represented graphically in Fig. 2.

Within the past two decades mathematical techniques (Coulson, 1952) have been devised which have permitted prediction of the wave length and in some cases the intensity of absorption bands due to electronic transitions in a number of simple molecules. Some larger, symmetrical molecules have also yielded to these techniques (e.g., benzene). The complexity of these calculations for more complicated molecules is staggering. It should be emphasized that for most molecules of biological importance good correlation between mathematical prediction and experimental fact has not been obtained.

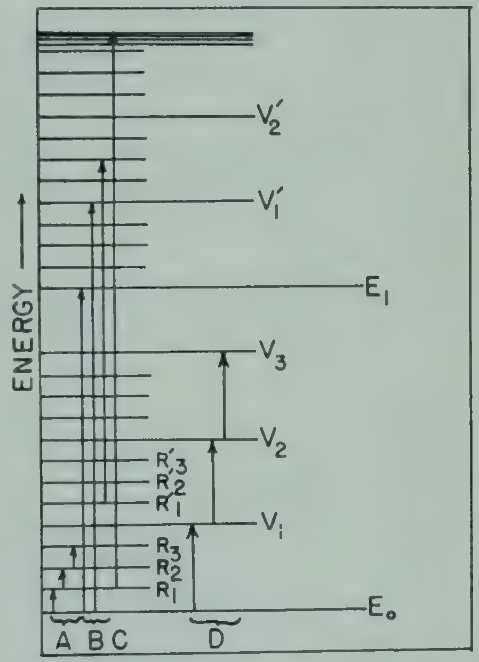

FIG. 2. Schematic molecular energy diagram. A. Rotational transitions. B. Combined electronic, vibrational, and rotational transitions. C. Transition leading to dissociation. D. Vibrational transitions.

The result of this unfortunate state of affairs has been a steadily growing body of empirical knowledge from which have come some generalizations supplemented by catalogues of spectral data. It is in fact this body of information that, at present, forms the basis for the most important application of absorption spectroscopy in biology—the application in qualitative and quantitative analysis.

III. Empirical Correlation between Molecular Structure and Absorption Spectra

From the foregoing brief discussion and from the much more detailed considerations by Hiskey (see Chapter 3) it may be seen that the prediction of the absorption spectra of complex molecules by quantum-mechanical calculations is under present circumstances impossibly complicated. For this reason and because the study of ultraviolet absorption spectra was already well under way before the quantum principles were widely understood, the correlation of ultraviolet absorption and molecular structure has proceeded on an almost completely empirical basis. This is especially true in the field of biology.

As the body of empirical data grew some correlations emerged in a nebulous form (See Ferguson, 1948 for a good account of the development of ideas in this field). These became somewhat better defined as ideas concerning the nature of the chemical bond and the concept of molecular orbitals developed. Soon some predictions of absorption spectra from a knowledge of molecular structure, and deductions in the opposite direction, became possible.

The correlation between the type of spectrum observed and its basis in molecular structure is to be considered first.

1. TYPE OF SPECTRUM

The absorption of a quantum of visible or ultraviolet energy by a molecule will result in the elevation of one or more of the bonding electrons to a higher energy level. In so doing, the configuration of the molecule alters and the nuclei of the atoms composing the molecule move further apart. It can be seen from the Morse curves (Chapter 5) that the molecule may (1) start from a number of different vibrational levels in the ground state, (2) find itself in a different vibrational state in the upper electronic state, and (3) may acquire sufficient energy to dissociate. With high energy radiation the molecule may lose one or more electrons to become an ion.

These possibilities would lead one to expect a series of absorption lines corresponding to each allowed transition, ending abruptly in a region of continuous absorption corresponding to the dissociation of the molecule. The beginning of the continuous absorption therefore corresponds to the energy of dissociation. The general location in the spectrum of the group of absorption lines should correspond to the energy of the electronic transition, and should be temperature-independent. The individual lines would represent the energy of the electronic transition modified by the vibrational energy and at least theoretically by the rotational energy. The relative intensity of these lines depends upon the number of times each particular transition is made during the period of observation (transition probability). This vibrational fine structure should be temperature-dependent.

Such a discrete line spectrum is in fact found in many organic compounds in the gaseous state at low pressures. In the solid state and in solution, the line spectrum is modified. It has been customary to divide the modified spectra into (1) diffuse spectra, corresponding to dissociation spectra, (2) multi-banded spectra, corresponding to a broadening of discrete lines into a continuous envelope of absorption out of which peaks of the stronger vibrational-electronic bands project, and (3) single banded spectra which represent a further broadening of the lines of the spectrum until these are reduced to a single broad band hundreds of Angstroms wide. Often the only remaining evidence of the discrete lines are one or more inflections of the otherwise smooth absorption envelope. The latter two types are but arbitrary divisions of a phenomenon of degree. Examples of each of these three types of spectra will be found in the following section.

The observed widths of absorption bands are dependent upon a number of factors both physical and instrumental. To the extent that these are significant in practical spectroscopy they will be considered under the appropriate headings. For more detailed accounts the interested reader is referred to the chapter by Hiskey in this volume, to Herzberg (1950), and to Lewis and Calvin (1939).

2. MOLECULAR GROUP SPECTRA

From the tremendous body of literature on absorption spectra there has emerged the fact that electronic spectra could be divided into two large groups, (1) spectra attributable to certain groups of atoms which were more or less independent of the rest of the molecule, and (2) spectra which were attributable to the molecule as a whole. The former case is a reflection of the fact in some molecules the electrons are restricted to the region of the nuclei of the atoms comprising the groups and are but little perturbed by alterations in the rest of the molecule. The second case reflects the situation that obtains when, by reason of conjugation, the electrons of the unsaturated bond become essentially the property of the entire molecule and therefore are perturbed by changes in any part of the molecule.

The locations of the so-called group spectra have been extensively catalogued and are summarized in Table I. Because these groups confer the property of absorption to the remainder of the molecule they have been called "chromophores."[2] It can be seen that the so-called chromophores may be composed of from two to many atoms.

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[2] The word "chromophore" should always be qualified by reference to the general region of spectrum referred to, such as "ultraviolet chromophore," "visible chromophore," since the use of the word has been extended beyond its proper etymological limits.

It should be emphasized that the distinction between the two types of spectra referred to above is not a fundamental distinction. The assignment of spectra to one or the other of the two categories may be on an operational basis. For instance, the absorption spectrum of benzene considered as a molecule would certainly belong to the second type, while the same ring structure when included in the molecule phenylalanine might well be considered a spectrum of the first type. For this reason the absorption spectra of benzene and other nuclei are included in the above table of group spectra. A second feature of Table I that should be emphasized is that the values given are approximations. This is because certain groups which in themselves may show little or no absorption in the ultraviolet can nevertheless exert a profound effect upon the position of the absorption band of a chromophore into which it has been substituted. Such substituents have been called auxochromes. In cases in which the effect of the auxochrome is particularly marked the resulting compound may be considered a new variety of chromophore. Here again the distinction is on an operational basis.

3. Modification of Group Spectra

Deviations in the position and intensity of the absorption of various chromophores have been studied extensively. As a result it is possible to correlate the deviations with certain types of structural alterations in the absorbing molecule. These correlations considerably extend the utility of Table I and therefore should be considered in some detail.

a. Coupling of Chromophores

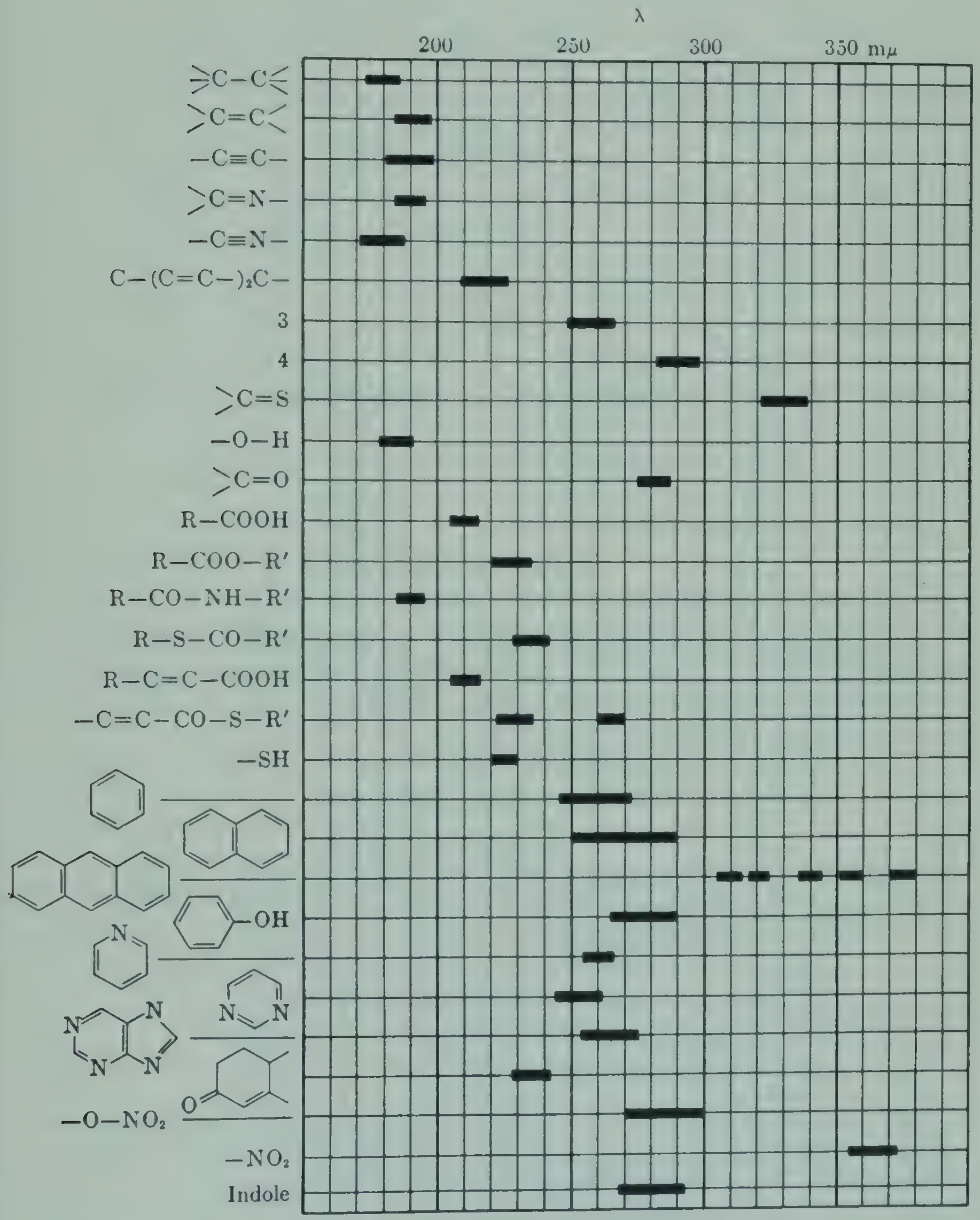
(1) When two chromophores are linked together in such a way that the electrons of each may fuse, a new chromophore is formed. This effect is seen for instance in the spectrum of naphthalene (Fig. 3). This figure shows clearly that the spectrum of naphthalene is not simply 2× the spectrum of benzene.

(2) When two chromophores are joined by saturated link this link acts esentially as an insulator for the π electrons and the two chromophores act independently. An example of this generalization is seen in Fig. 4. Here one sees that the spectrum for diphenylethylene on the molecular absorbancy basis is quite closely represented by 2× methylbenzene.

(3) When two chromophores are joined by a conjugated system the absorption of the individual chromophores is altered. The degree to which the spectrum is altered is often quite large and it is frequently difficult to distinguish in the spectrum any part of the absorption of the original chromophores. Some cases in this group have been investigated systematically

TABLE I

The approximate position of the absorption bands of some commonly occurring chromophores



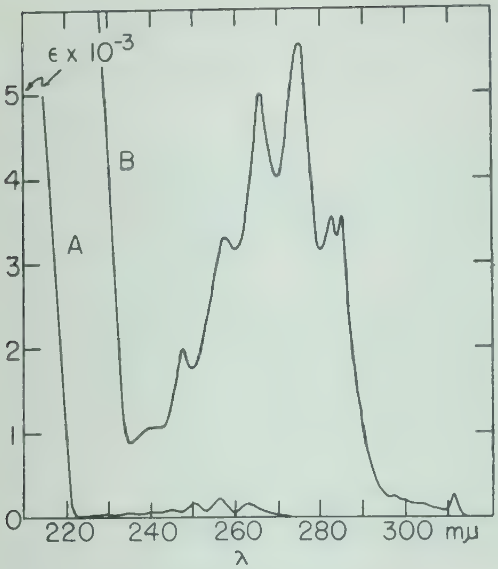


FIG. 3. A. Benzene. B. Naphthalene.

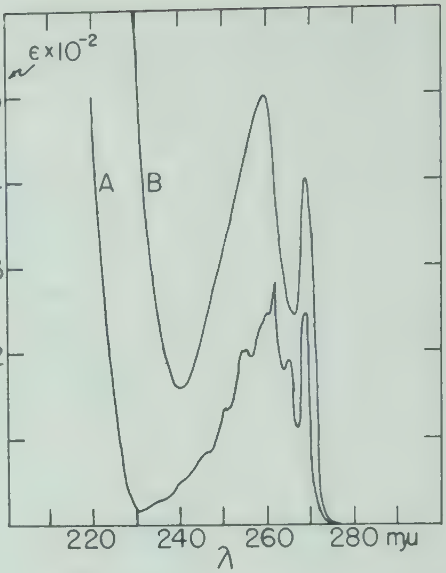
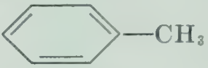
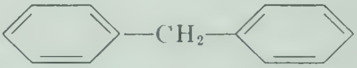


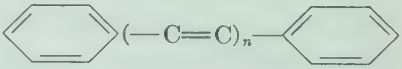
FIG. 4. A.



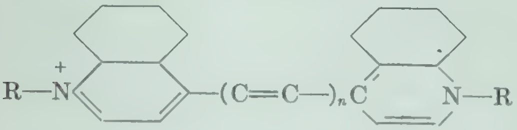
B.



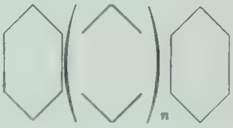
(see Lewis and Calvin 1939). In these studies it was possible to demonstrate two types of relation between the position of the first absorption maximum and the length of the conjugated chain joining the two chromophores. First, for a series of compounds of the type



the relation $\lambda^2_{1^{st}\,max} = kn$ has been demonstrated to hold as is shown in Fig. 5. Second, in the systems



and



the linear relation $\lambda_{1^{st}\,max} = k'n$ has been demonstrated. This is illustrated in Fig. 6.

The straight lines drawn through the points in the figures given above represent the predicted result derived from the treatment of these molecules as linear oscillators. The analogy between a conjugated system and a linear oscillator has been called on to provide a framework for understanding the difference in the absorption spectra of certain stereo isomers.

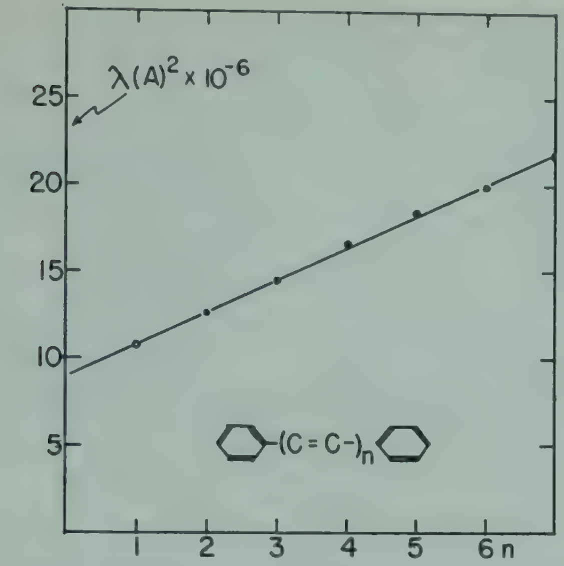


FIG. 5. Redrawn from Lewis and Calvin (1939).

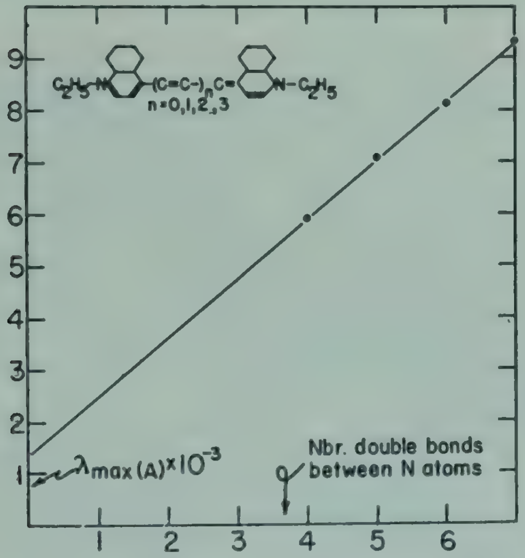
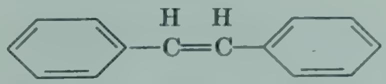


FIG. 6. Redrawn from Lewis and Calvin (1939).

This is best illustrated by the stilbenes



(Roe and Beall, 1953). When the three dimensional model of this molecule is examined (constructed on the basis of x-ray diffraction) it can be seen, as shown in Fig. 7, that the compound can have either the *trans* (A) or

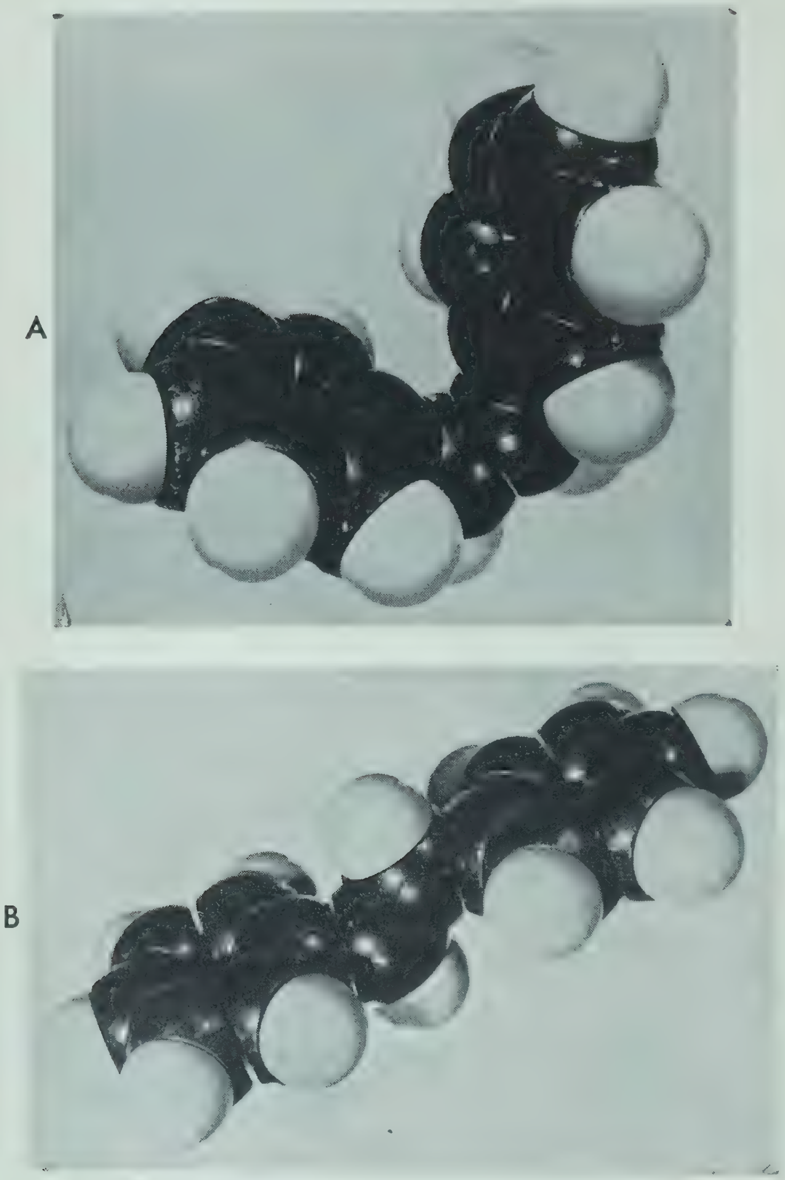


FIG. 7. Models of stilbene: A. *cis*. B. *trans*.

the *cis* (B) configuration. The spectra for these two isomers of stilbene are shown in Fig. 8.

Any polyene chain has the possibility of a definite number of stereoisomers.[3] In such cases the calculation based on the linear oscillator analogy predicts that the strongest band and the lowest value of the wave length of

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[3] This number is $(2)^n$ where n is the number of (—C═C) groups.

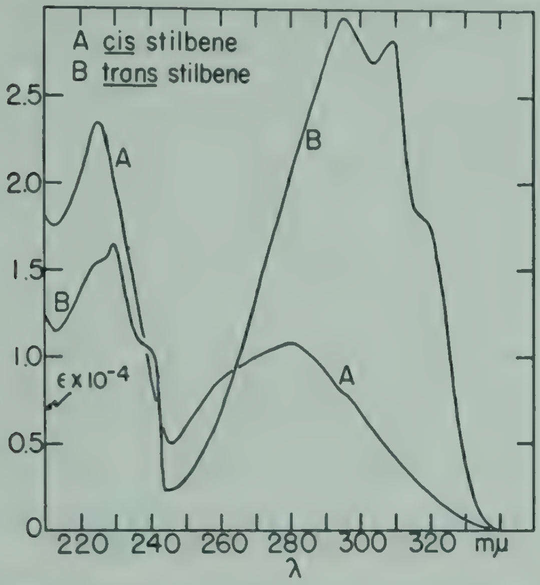


Fig. 8. Redrawn from Roe and Beale (1953).

the first maximum would be found by the isomer with the most nearly linear electron path. The spectra of stilbenes and of certain carotenoids demonstrate this (Zechmeister and Pinckard, 1947).

The degree of co-planarity of the conjugated chromophores of the *trans* type may be disturbed by bulky substituents. This is clearly reflected in the spectra of the methyl substituted *trans* stilbenes (Beale and Roe, 1952). Models of these compounds are shown in Figs. 7A, 9*a* and the spectra in Fig. 9*b*.

(4) Although most compounds of the structure described by (2) above do indeed behave as though their chromophores were independent, there are

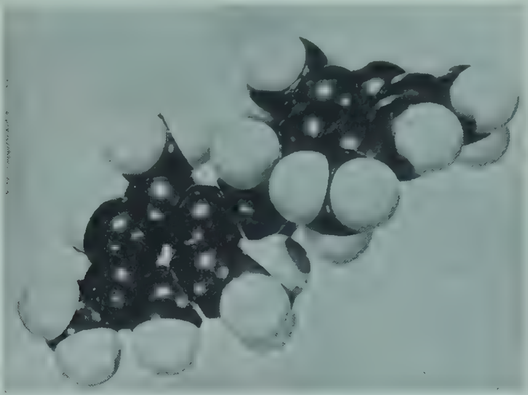


Fig. 9*a*. Model of stilbene: 2,4,6,2′,4′,6′-hexamethylstilbene.

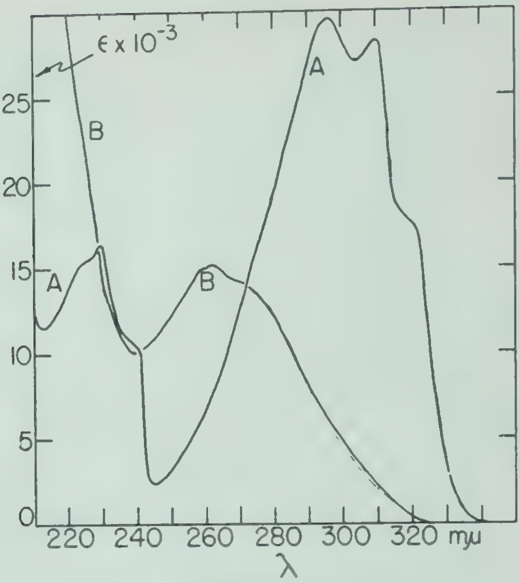


FIG. 9*b*. A. *trans* stilbene; B. 2,4,6,2′,4′,6′-hexamethylstilbene. Redrawn from Beale and Roe (1952).

some compounds of that general configuration which show distinct spectroscopic evidence of interaction between the chromophores. The generalization that covers this class of alterations may be stated as follows: when two resonating chromophores are connected by an insulating link of sufficient length as to permit close approximation of the resonators to one another, it is possible for the π electron clouds of the two to interact and thus alter the spectrum of both chromophores. The configuration favoring interaction may be of lower energy than other configurations (Weber, 1950). Two clear examples of this phenomenon drawn from the biological literature are demonstrated in Figs. 10 and 11. This phenomenon may be of greater than analytical significance in biology and will be discussed again later in the chapter.

b. Tautomerization

It sometimes happens that the formal structure written for certain compounds indicates the presence of a chromophore when in fact such a chromophore is not found spectroscopically. This is usually taken as indicating that a tautomeric structure for the compound is probably a predominant form. Indeed, spectroscopic evidence may be extremely valuable in establishing the fact that certain tautomeric structures do exist, and in demonstrating the relative predominance of one or the other of the tautomeric forms for a certain set of conditions. Such information is frequently of im-

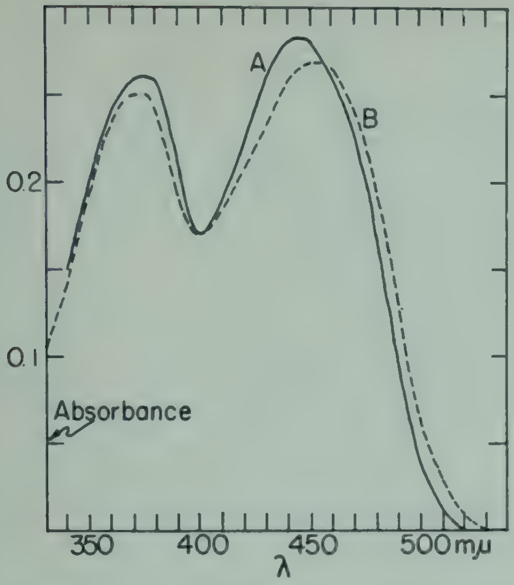


FIG. 10. A. Riboflavin; in water. B. Riboflavin in water solution of adenosine, 0.05M. Redrawn from Weber (1950).

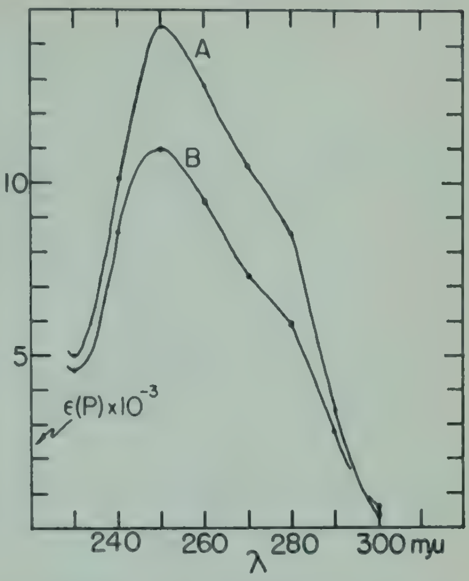
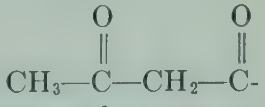
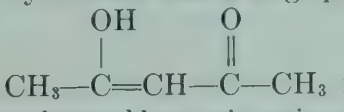


FIG. 11. A. Desoxyguanylic mononucleotide. B. Desoxyguanylic dinucleotide. Data of R. L. Sinsheimer; private communication.

portance when considering reactions in which the compound may participate and in understanding certain of its physical and biochemical properties.

The following examples will serve to illustrate the spectroscopic changes which accompany tautomerization as it occurs in a few classes of compounds.

(*1*) *2,4-diketones.* The spectrum of acetyl acetone  $CH_3—\overset{\overset{O}{\|}}{C}—CH_2—\overset{\overset{O}{\|}}{C}—CH_3$ might be expected to be essentially the same shape as the spectrum for acetone with twice the absorption intensity considered on a molar basis. This comparison is made in Fig. 12. The striking discrepancy between the spectra may be explained by the fact that a large portion of the compound is actually in the form  $CH_3—\overset{\overset{OH}{|}}{C}=CH—\overset{\overset{O}{\|}}{C}—CH_3$ in which the carbonyl absorption is considerably enhanced by conjugation with the enolic —C=C. The addition of alkali would be expecetd to stabilize the enolic form by salt formation with the enolic hydroxyl. This effect is demonstrated in Fig. 12.

If by adjustment of the pH one obtains the spectrum of essentially pure ketonic form and of essentially pure enolic form, it will then be possible to calculate from spectral data the position of equilibrium between the two

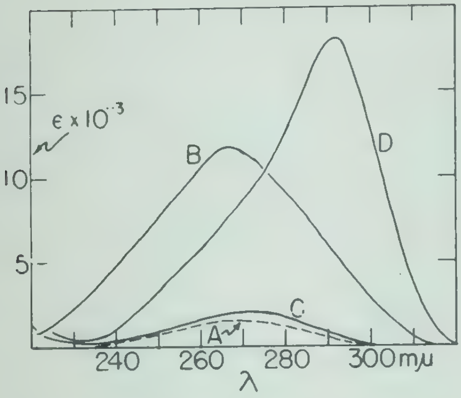


FIG. 12. A. Acetone in hexane (ordinate values multiplied by 100 before plotting). B. Acetylacetone in hexane. C. Acetylacetone in water. D. Acetylacetone in 0.01N NaOH. Redrawn from Henri (1932).

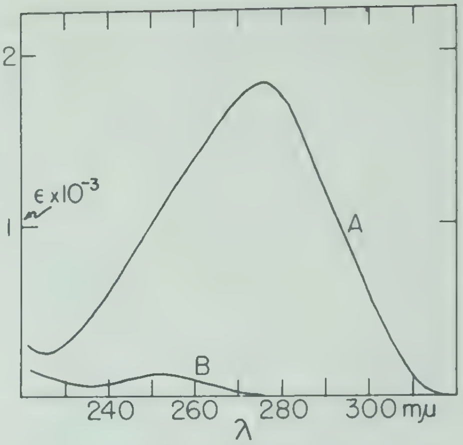
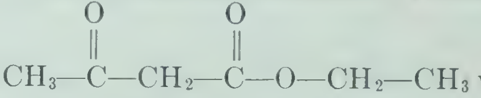


FIG. 13. A. Acetylacetone in water. B. ethyl acetoacetate in water. Redrawn from Henri (1932).

forms over a wide range of conditions. The technique for handling such problems is discussed in detail in Chapter 3.

(2) *Keto acids and esters.* From the preceding discussion it might be expected that ethylacetoacetate  $CH_3—\overset{\overset{O}{\|}}{C}—CH_2—\overset{\overset{O}{\|}}{C}—O—CH_2—CH_3$ would show a similar phenomenon. The data in Fig. 13 show that this is not the case. The fact that this compound has two stable tautomeric forms is suggested by the boiling point behavior. If one constructs a model of the molecule allowing the appropriate bond angles and van der Waal's radii it is seen that the atoms may be so oriented that the enolic hydrogen and the carboxyl carbonyl approach one another in such a way as to allow the formation of an internal hydrogen bond. The formation of this hydrogen bond has the effect of reducing the double bond character of the carbonyl group, which in turn yields the observed spectral change. This form of the compound will also have the lower boiling point (Fieser and Fieser, 1944, p. 315).

Thiol esters (Lynen, 1953) are becoming increasingly important in an understanding of some metabolic pathways. The function of coenzyme A in fatty acid metabolism is a good example. Compounds of the thiol ester class show some interesting and important spectral changes accompanying the tautomeric shift within the molecule. Figures 14 and 15 are examples of such alterations.

(3) *Acids and aldehydes.* Unconjugated acids and aldehydes might be

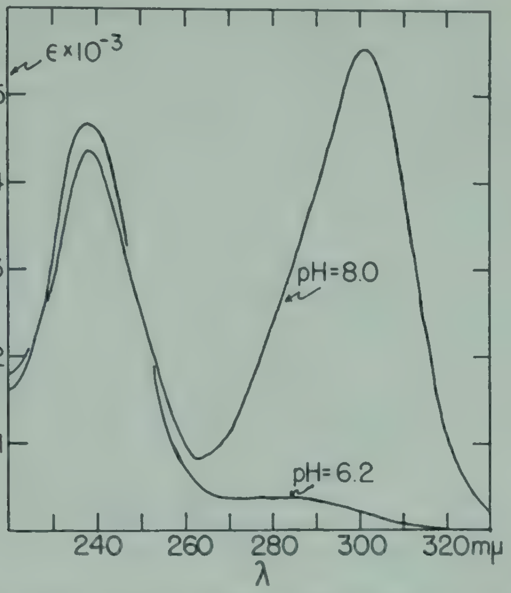
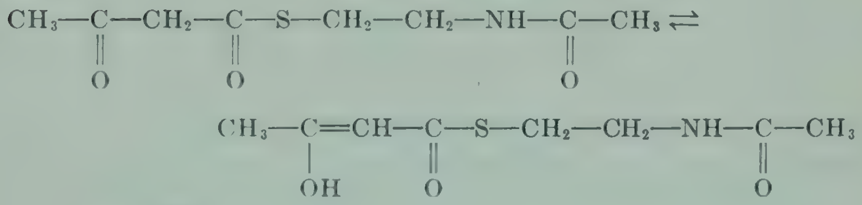


FIG. 14.



$$\mathrm{CH_3{-}\underset{\underset{O}{\|}}{C}{-}CH_2{-}\underset{\underset{O}{\|}}{C}{-}S{-}CH_2{-}CH_2{-}NH{-}\underset{\underset{O}{\|}}{C}{-}CH_3} \rightleftarrows$$

$$\mathrm{CH_3{-}\underset{\underset{OH}{|}}{C}{=}CH{-}\underset{\underset{O}{\|}}{C}{-}S{-}CH_2{-}CH_2{-}NH{-}\underset{\underset{O}{\|}}{C}{-}CH_3}$$

Redrawn from Lynen (1953).

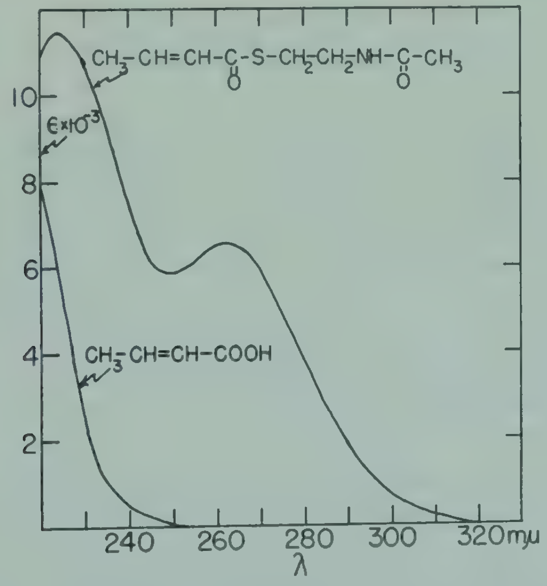
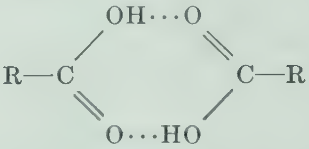
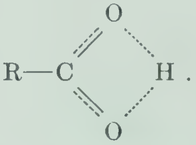


FIG. 15. Redrawn from Seubert and Lynen (1953).

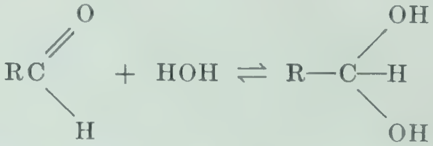
expected to have absorption spectra much like that of acetones since the $\rangle\mathrm{C{=}O}$ bond is found in all. When compounds belonging to these groups are dissolved in polar solvents ultraviolet absorption bands attributable to the $\rangle\mathrm{C{=}O}$ group are either weak or absent. There are at least three well defined phenomena which would explain these observations. First, two molecules may associate head to head



or, second, a single molecule may take the form



Third, in the case of aldehydes, the molecule may actually react with water taking on one mole of water to form a hydrate



The first two of these phenomena are common in organic acids and result in the reduction of the double bond character of the C=O group. The reaction with water is seen in aldehydes and is demonstrated in Fig. 16. While the formation of hydrates is not a tautomerization in the true sense of the word, it has been included since the resulting diol is similar to some of the tautomeric forms. The influence of the nature of the solvent upon the structure of compounds anticipates to some extent the discussion of environmental influences on absorption spectra.

(*4*) *Amide absorption.* The subject of absorption due to the amide group has been the center of controversy for a number of years. It would be well to start with the least controversial part of this subject: the absorption of the simple amide linkage. Recently Saidel and his coworkers (Goldfarb and Saidel, 1951; Saidel *et al.*, 1951a, b) have studied the absorption of such substances as glycylglycine, and diglycylglycine with a vacuum ultraviolet

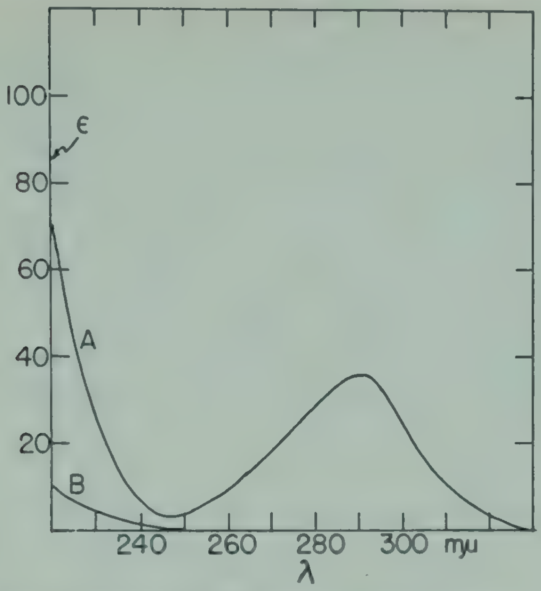


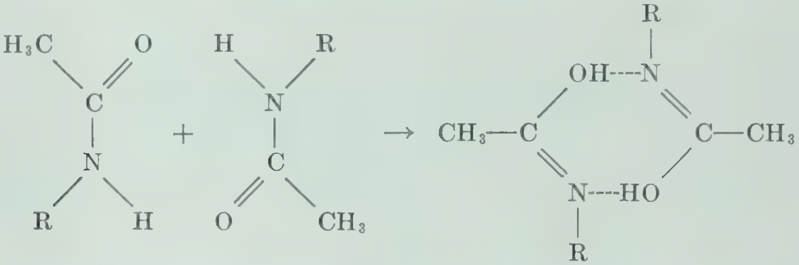
FIG. 16. A. Chloral in hexane. B. Chloral hydrate in water. Redrawn from Brode (1943).

spectrograph. An absorption maximum was found for these compounds and for bovine serum albumin in the vicinity of 1900A and it was concluded that this absorption was associated with the peptide linkage. They also examined a larger series of compounds with the Beckman DU photoelectric spectrophotometer in the region of the low wave length limit of the usefulness of the Beckman, 2050A in this case. They concluded that the $E^{2050\text{A}}_{\text{peptide bond}}$ was 2800 ± 500. In connection with spectrophotometery in this portion of the spectrum see the section on "Instrumentation" for errors.

These results by Saidel are certainly consistent with the general body of information on the absorption characteristics of simple carboxylic acids and amides in the vicinity of the beginning of the vacuum ultraviolet (see Table I). It is also consistent with the evidence for a partial double bond character for the amide linkages from the x-ray diffraction studies of simple dipeptides (Pauling *et al.*, 1951). (Compare C—N and C═N in Table I).

The absorption of the peptide bond has also been studied recently by Setlow and Guild (1951). These workers conclude that this bond is the major absorbing structural unit in the protein molecule below 2300A, and that the absorption of the peptide bond extends as a toe as far up as 2700–2800A. The contribution of this absorption to the total absorption of the protein at 2537A is significant. This latter fact is of some importance in studies of the effect of ultraviolet radiation on proteins. (See Chapter 1).

Similar studies of peptides and proteins by Anslow and her group (see for example Anslow and Nassar, 1941; Anslow, 1945), led them to propose that the absorption by globular proteins in the vicinity of 2800A was due primarily to the amide linkage and not due as is more widely believed to the aromatic nuclei in the amino acid residues. In the case of the fibrous proteins Schauenstein (1950) and his coworkers have studied the ultraviolet absorption in both ordinary and plane polarized radiation and have come to the conclusion that in such proteins there is absorption arising from an extensive network of hydrogen bond cross links between adjacent polypeptide chains involving the adjacent amide groups. This hydrogen bonding would convert the —N—C═O to —N═C—O· · · since the enolization is required for the formation of the type of structure which is represented by the dimerization of *N*-alkyl amides through hydrogen bonds.



This type of structure is thought to be the chromophore of the fibrous protein which gives rise to a very broad absorption band with a maximum in the vicinity of 2500A. This band demonstrates a dichroism in silk fibroin oriented at 40° to the fiber axis and at 50° in the case of collagen.

The work of Anslow and Schauenstein has been recently reviewed by Beaven and Holiday (1952) who adduced much evidence highly critical of the conclusions and to some extent of the data. Beavan and Holiday arrive at the conclusion that with two notable exceptions, the work of Anslow is completely at variance with both the theory of the absorption of the chromophores involved in the peptide link and with the known absorption of the residue chromophores. They note that much of the contrary evidence adduced by them in connection with the work of Schauenstein is based on observations on globular proteins, and that the dichroism of the chromophore of the cross bonding observed by Schauenstein remains as the best evidence for his proposals. They point out that relatively little use has been made of ultraviolet dichroism, and they consider this tool to be of great potential in the study of protein structure.

c. Environmental Factors

In the previous section some empirical correlations between molecular structure and absorption spectra have been presented. Modification of these correlations due primarily to changes within the molecules themselves and more or less independent of the surroundings were discussed. It has been pointed out, however, that the absorption spectra of an assemblage of molecules is frequently influenced strongly by factors inherent in the environment. Some of these influences are now considered.

(*1*) *Solvent.* The absorption spectrum of a molecule is altered when it interacts in any of a number of ways with other molecules. It may intereact with like molecules as in the case of a pure liquid or solid, or with unlike molecules such as solvent molecules in the case of solutions. In passing from a gas at low pressure to a gas at high pressure and thence to a liquid, the absorption spectrum of a compound departs increasingly from that which might have been predicted. The extent to which the absorption spectrum of a molecule in solution is altered depends upon the extent to which the solvent interacts with the absorbing molecule, and/or the solute interacts with itself (Klotz, 1947; Scheibe, 1937, 1925; Blout *et al.*, 1946).

The interaction may vary from weak van der Waal's type of interaction to actual compound formation. Similarly the magnitude of the solvent effect upon absorption spectra varies considerably, manifesting itself only as a broadening of the vibrational fine structure when there is but little interaction or as a shift of the absorption band to a distant portion of the spectrum in the case of compound formation (see Fig. 16).

It is thought that the effect of polar solvents upon the absorption spectrum of certain compounds is due to an association of the polar portions of the solvent molecule with polar groups of the chromophore. It might be predicted that the effect of alcohol on the absorption spectrum of a chromophore dissolved in hexane would not be directly proportional to the mole fraction of alcohol present, since the alcohol molecules might tend to aggregate about the polar groups of the chromophore molecules in the hexane solution. This effect is not uncommon and is demonstrated in Fig. 17. Such a graph should certainly be prepared in any case in which mixed solvents are employed. An early attempt to generalize the relation between the nature of the solvent and its effect on the absorption spectrum of the solute was made by Kundt (1878). Kundt's law stated that of two solvents, the one of greater refractive index and dispersion would shift the wave length of the absorption maximum to a longer wave length than the solvent of lesser value for these parameters. This relation has been found to hold in only a limited number of cases and therefore is not useful (Herbert, 1933). (See also Ferguson, 1948; Brode, 1943, p. 185; Spears, 1928.)

(*2*) *pH*. Under the general discussion of chromophores, the contribution of ionic forms to the absorption spectrum is discussed in Chapter 3. Certain chromophores possess ionizable groups which participate directly in the electron system giving rise to the absorption of ultraviolet or visible light. In compounds of such structure the degree to which the population of molecules is ionized will influence strongly the absorption spectrum. This

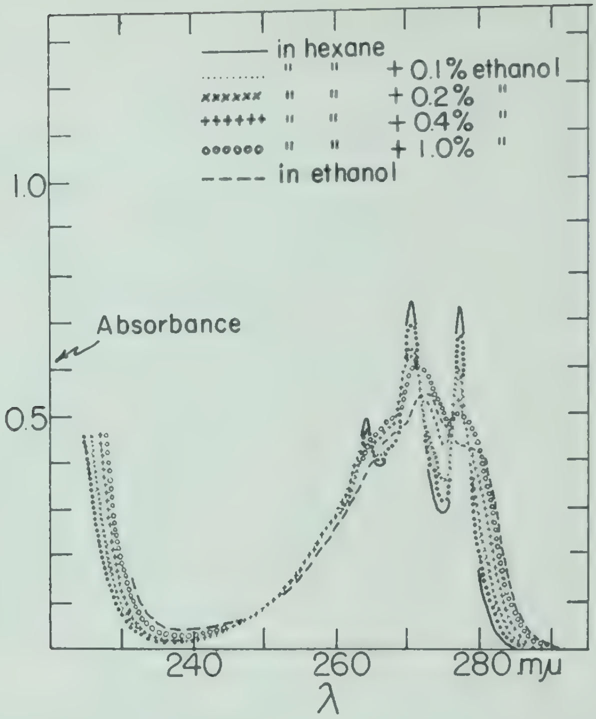


FIG. 17. Phenol in pure and mixed solvents.

effect is best known in the visible indicator dyes but is by no means limited to the visible spectrum. For example, the effect of pH on the absorption spectrum of uracil is illustrated in Fig. 18. The presence of the isosbestic points A and B (see Chapter 3, p. 97) suggests that the change in the absorption spectrum is due to an alteration in the position of equilibrium between two forms that might be represented by the structures given in Fig. 18. By plotting $\lambda_{\min}$, $\lambda_{\max}$, or intensity at some wave length *vs.* pH one may determine the pK of uracil or any other compound of similar behavior. This procedure is illustrated for uracil in Fig. 18. The details of the calculation for equilibrium and ionization constants are dealt with in Chapter 3. It should be pointed out, however, that the use of absorption spectrophotometry in study of equilibrium reactions has yielded valuable information since it is one of few convenient and sensitive methods by which the position of equilibrium may be determined rapidly and accurately without altering significantly the reaction.

The effect of pH on the spectrum has been of great importance in analytical applications of absorption spectroscopy. In assigning structure to an unknown compound the agreement of the spectrum of the unknown compound with that of a known compound over a wide range of pH values lends great weight to the assignment. This procedure has been used a num-

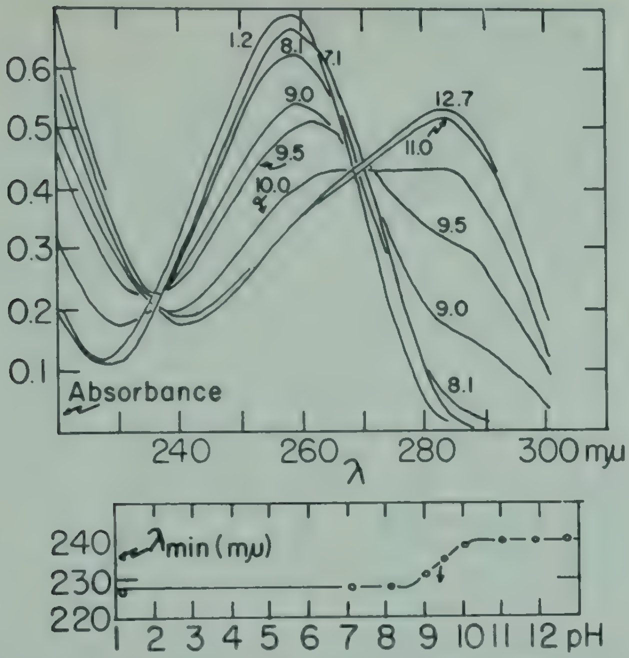


FIG. 18. Absorption spectra of uracil at various pH's and an example of the spectrophotometric estimation of pK_a. pK_a, spectrophotometric estimation, 9.4; found by titration, 9.45.

ber of times to identify unknown compounds isolated from biological systems. Figure 19 gives a series of spectra which were of great assistance in an instance of structure determination of importance to biology.

In Chapter 3 spectrophotometric analysis of two-component systems was discussed and the factors affecting the accuracy of the analysis were considered. In instances in which there is little difference in the absorption spectra of two or more compounds at a given pH, there may be other pH regions in which the difference is much greater. It is clear that for quantitative analysis it is important to determine whether the absorption spectrum varies with pH and, if it does, the details of this variation. These facts are important in establishing the conditions of spectrophotometry to yield the maximum sensitivity and the minimum error, or, if necessary, the optimum compromise.

Consider for example the spectrum of uracil in Fig. 18. It is immediately clear that the dA/dpH is zero at the isosbestic point and hence the error dC/C due to small changes in pH would also be zero but the sensitivity is approximately $\frac{\max}{3}$ and the $dA/d\lambda$ is high. It is to be noted that on the acid side of neutrality the dA/dpH is very low and the sensitivity is at a maximum at $\lambda_{\max}$. Furthermore, in the immediate vicinity of $\lambda_{\max}$ $dA/d\lambda$

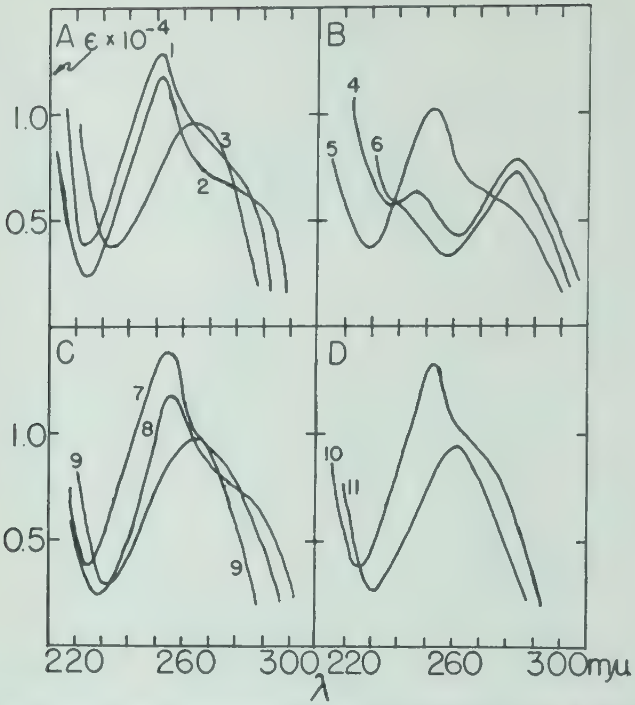


FIG. 19. Spectroscopic evidence supporting the assignment of the ribosyl linkage to position 9 in guanosine. A. 9-methyl guanine; B. 7-methyl guanine. C. Guanosine. D. Guanine desoxyriboside. 1,4,7, and 10 in water. 2,5, and 8 in N/20 HCl. 3,6,9, and 11 in N/20 NaOH. Redrawn from Gulland (1938).

is very low, hence the errors due to small uncertainties in wave length and pH are at a minimum other than zero, while the sensitivity is at a maximum.

A thorough discussion of the assessment of error is given in Chapter 3 but this example will illustrate the role of pH in the error calculation. Other situations are more complicated than the one illustrated, but the principle remains the same.

(3) *Redox*. The capacity for reversible oxidation and reduction is found in many compounds of biological importance, for, in fact, such reactions are the basis of storage and release of energy by biological systems. The oxidation and reduction of such compounds is usually accompanied by a fundamental alteration in the π electron system, resulting in a distinct alteration in the absorption spectrum of the compound.

An excellent example of the use of the redox effect is illustrated in Fig. 20, which gives the absorption spectrum of the oxidized and reduced form of diphosphopyridine nucleotide (DPN) (coenzyme I). A number of workers have designed enzyme assay reactions in such a way as to make the oxidized or the reduced form of DPN the limiting reactant, then by measuring the change in the absorption at appropriate wave lengths have determined reaction rates. Figure 21 gives an example of such a determination.

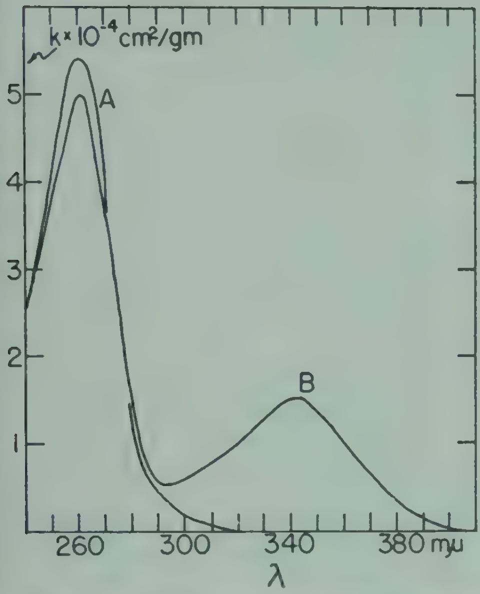


FIG. 20. Diphosphopyridine nucleotide: A. Oxidized. B. Reduced. Redrawn from Warburg and Christian (1936).

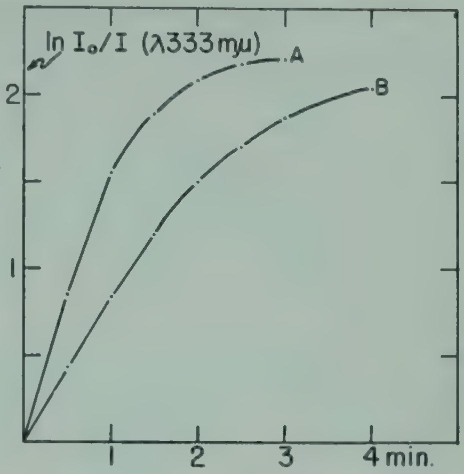


FIG. 21. The rate of reduction of diphosphopyridine nucleotide by lactic acid dehydrogenase as measured by the increase in absorbance at 333 mμ (see Fig. 20). A. 0.78 μg protein/ml. B. 0.39 μg protein/ml. The purity of the enzyme preparation is estimated by expressing the initial rate of reduction of the nucleotide in terms of the concentration of protein in the reaction mixture. From data given by Kobowitz and Ott (1943).

Some compounds or tautomeric forms of them can be reduced by molecular oxygen. It is good practice to measure the absorption spectrum of a solution before and after vigorous shaking, if one has reason to believe that the substance in solution is undergoing spontaneous oxidation. The effect of this procedure on the absorption spectrum of ascorbic acid is demonstrated in Fig. 22. As in the case of the pH effect the oxidation and reduction of compounds can, when controlled, be of analytical significance, or when uncontrolled can result in serious errors.

(4) *Temperature.* In the short section on the theory of light absorption use was made of diagrams of energy states of molecules (Fig. 21). At room temperature essentially all molecules are in the lowest electronic (E_0) level but are distributed in the vibrational-rotational levels in a manner which depends upon the absolute temperature (Maxwell-Boltzmann distribution law). When a molecule absorbs a quantum of visible or ultraviolet energy it undergoes a transition to one of the upper electronic levels where it may occupy one of the vibrational-rotational levels. The difference in energy between the initial and final levels is the energy and hence, since $\Delta E = h\nu$, the frequency of the absorbed photon. The number of energy levels occupied in the E_0 state increases as the temperature of the substances in-

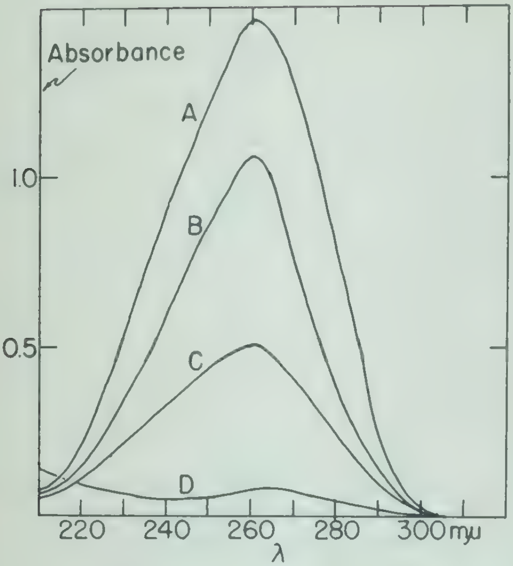


FIG. 22. Oxidation of ascorbic acid by molecular oxygen. Ascorbic acid 36 mg./liter water shaken vigorously in contact with air. A. Zero time. B. 1 min. C. 3 min. D. 5 min.

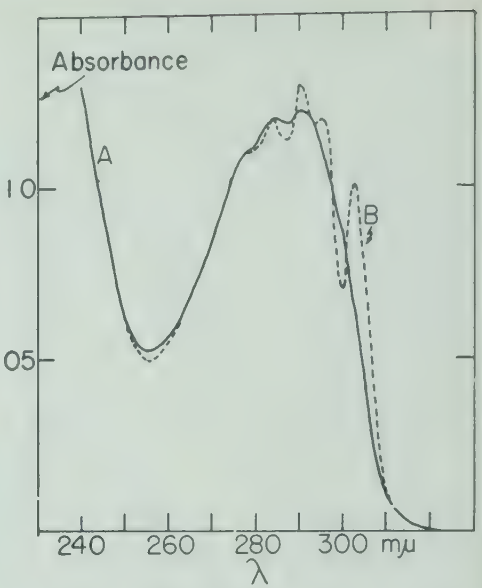


FIG. 23. Tryptophan as a thin sublimed film. A. 298°K. B. 77.6°K. Sinsheimer and Scott, unpublished data.

creases, hence the number of possible transitions likewise increases, and the spectrum may become more diffuse. As the temperature is lowered a larger and larger percentage of the molecules in the E_0 state will occupy the lowest vibration-rotational level. This reduces the number of possible transitions and results in greater detail in the spectrum. This effect is illustrated in Fig. 23. Techniques for carrying out low temperature ultraviolet studies are reviewed and described in the reference from which this figure is taken (Sinsheimer *et al.*, 1950).

The situation is somewhat more complicated than the preceding paragraph might suggest. Interested readers may find a summary of the details in Sinsheimer *et al.* (1950) and Scott *et al.* (1952), which also contain numerous references to the original literature on this subject. As a matter of fact, it is these complicating factors that have restricted the use of low temperature spectra to relatively few special cases. It is, however, a potentially powerful tool and its application has been made considerably easier with the development of low temperature (liquid nitrogen or helium) attachments for the automatic recording spectrophotometers (Scott, unpublished; Passerini and Ross, 1953).

In more special applications low temperature has been resorted to not so much for its specific effect upon spectral details but because at low temperatures it has been possible to create and identify compounds which under ordinary conditions would have been of too short a mean life to be detected. This particular type of application has not been much used in biological work but deserves consideration (Lewis *et al.*, 1942; Lewis and Kasha, 1944).

Within the temperature range of the ordinary laboratory environment the magnitude of the temperature effect is small (see section on calibration) and need be considered only in cases where molecular complexes are contributing significantly to the spectrum observed. Commercial and laboratory equipment for temperature control is described in the section on "Instruments."

(*5*) *Ionic strength.* The effect of ionic strength is difficult to predict. It is small in the case of simple ions and small molecules. There are certain ions, e.g. borate, (Zittle, 1951; Blout and Asadourian, 1954) that exercise more than average effect on the spectrum. Macromolecules with a large number of charged groups such as proteins and nucleic acids may be strongly affected by ionic strength because the change in ionic strength undoubtedly changes the molecular configuration (Blout and Asadourian, 1954; Doty and Edsall, 1951) and hence the distribution of charge in the vicinity of the chromophore. The change in the shape of the molecule may also change the spatial arrangement of two or more adjacent chromophores, giving rise to an effect which is described more fully below. In Fig. 24 the effects of Na^+Cl^- and borate on the absorption spectrum of desoxypentosenucleic acid are compared.

The use of solvents of moderate to high ionic strength is common in biological work. In all such cases care must be exercised to ascertain the error which would be introduced by variation in the ionic strength, and calibration of the system should take the ionic effect into account.

(*6*) *Concentration.* The effect of concentration on the absorption spectrum of solute molecules has been long recognized. The basis for this behavior has been found primarily in the formation of complexes between the solute molecules. The tendency to form such complexes is a very variable property and further is often strongly influenced by the nature of the solvent (Sheppard, 1942; Sheppard and Geddes, 1944). From the standpoint of the biologist this particular facet of spectroscopy deserves careful study on two counts. First, there is some evidence (Arnold and Oppenheimer, 1950) that the formation of molecular complexes is a phenomenon of importance in itself in biology and, second, each particular system with which the biolgist deals by the method of absorption spectroscopy must be examined empirically to determine the degree to which results are a function of the concentration.

The concentration effect most commonly met with is that usually characterized as "failure of Beer's law" (see Chapter 3 for discussion of Beer's law). This connotation arises from the fact that if observations of light ab-

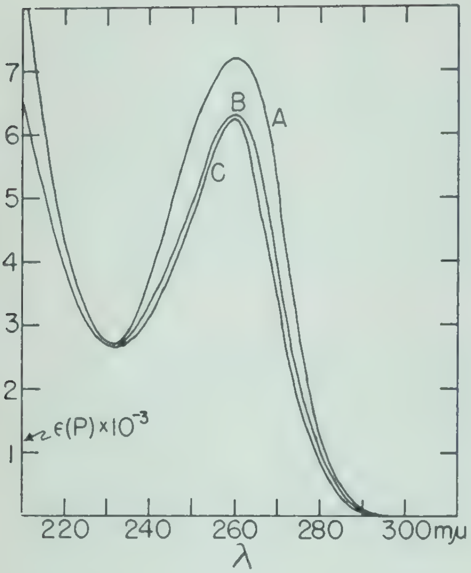


FIG. 24. Comparison of the effect of phosphate and borate ions on the absorption spectrum of desoxypentosenucleic acid. Nucleic acid dissolved in: A. Distilled water. B. 0.1 M phosphate buffer, pH 7.0; C. 0.005 M Borate buffer, pH 7.0. Scott and Wiegand, unpublished data.

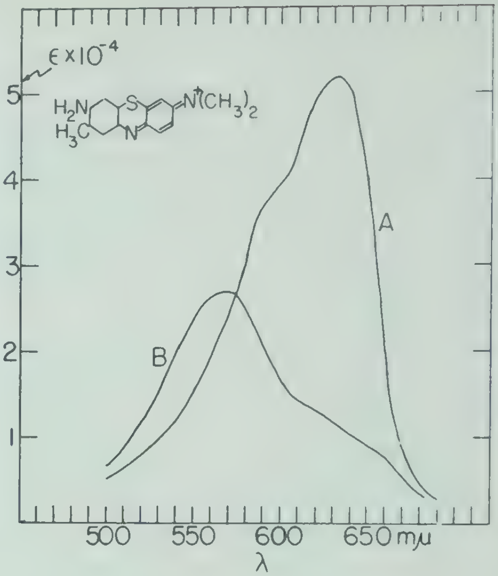


FIG. 25. Toluidine blue in water: A. 5.17×10^{-7} M; B. 3.88×10^{-4}. Redrawn from Michaelis (1947).

sorption are restricted to one band of wave lengths then the linearity of the relation between solute concentration and light absorption predicted by Beer's law may be found to hold only over a limited range of concentration. The cause of this deviation may be inferred if the complete absorption spectrum is determined at various concentrations. Figure 25 shows a familiar case in dye spectroscopy. It is clear that at concentration A there is a different average molecular population present than at concentration B. Physical chemical studies (Michaelis, 1947) have shown that this is due to the formation of a dimer or a polymer of the dye molecules, and that the relative proportion of dimer or polymer present is a function of the concentration of the monomer.

The exact mechanism by which the dye molecules aggregate is not known nor is it assumed to be the same in all cases (Scheibe, 1925, 1938; Scheibe *et al.*, 1943; Scheibe and Fauss, 1952; Pfeiffer, 1936; Jelley, 1937). It is clear from the absorption spectra that there is an alteration in the π electron system of these conjugated molecules.

Scheibe studied the dye pseudoisocyanine extensively, and showed that the material actually formed polymers of great length and that these were not random aggregations, but possessed a specific organization. In these high polymers Scheibe demonstrated the existence of a new absorption band which was not found in the spectra of the monomeric form of the dye. This absorption band he attributed to an electronic transition arising from the formation of electron orbitals which extend over more than one monomeric unit and which were oriented normal to the planes of the monomers. Essentially a new electronic species had been formed.

The alteration of the spectrum of chromophores by aggregation is by no means limited to dye molecules. It has been shown, for instance, that the spectra of high polymers of desoxyribose- and ribosenucleic acids are not the simple sum of their respective monomeric units. This is illustrated in Fig. 26. It has also been shown (Blout and Asadourian, 1954) that the absorption spectrum of highly polymerized desoxyribosenucleic acid itself changes with concentration. It has also been shown that for simple substances such as guanine there is a large change in the ultraviolet absorption spectrum as the amorphous solid crystallizes (Scott *et al.*, 1952). Similarly the absorption spectrum of the dinucleotide of guanine desoxyriboside phosphate is about 25 per cent lower than the sum of two monomeric nucleotides (Sinsheimer, 1954). See Figure 11.

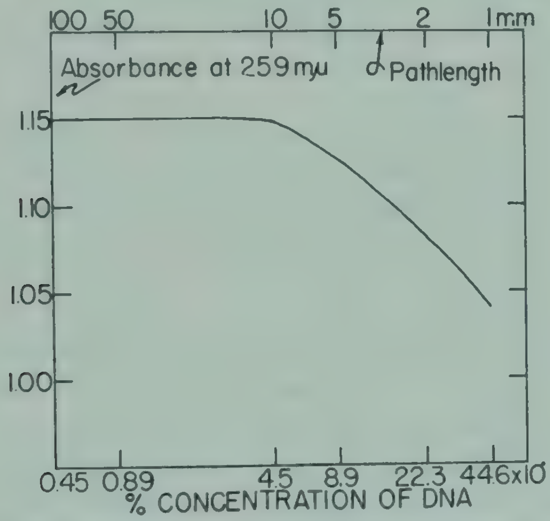


FIG. 26. Redrawn from Blout and Asadourian (1954).

(7) *Irradiation.* The energy absorbed by a molecule must be dissipated by the molecule. This may occur in a number of ways (collision, fluoresence, internal conversion, etc.) which result in the return of the molecule to the ground state without alteration in the bond arrangement within the molecule. Phenomena associated with the return to the ground state are of little significance in connection with the determination of the absorption spec-

trum, although fluoresence may introduce errors in spectrophotometry. The mechanisms for the loss of absorbed radiation are considered in detail in Chapter 1.

Phenomena associated with photochemical alteration are not uncommon in biological material, especially in connection with the absorption of ultraviolet radiation. In preliminary work with any problem involving ultraviolet spectrophotometry one should ascertain by consecutive measurements of the spectrum of the same sample that the amount of energy absorbed during a determination does not alter the spectrum by an amount greater than the overall error in the measurement.

IV. Instrumentation

1. INTRODUCTION

The material covered in this section will be restricted as far as possible to instruments designed primarily for absorption spectroscopy in the ultraviolet portion of the spectrum. It is of course true that all such instruments can to some extent be used in the visible region and vice versa. In all cases the discussion of the use of any instrument will be limited to ultraviolet absorption spectroscopy.

In the past decade the commercial availability of photoelectric ultraviolet spectrophotometers has changed remarkably. Prior to that time the majority of the spectrophotometers were photographic instruments. Earlier volumes concerned with absorption spectroscopy as a method contain chapters on ultraviolet spectrophotometry in which much space is given over to an extensive and detailed consideration of light sources, monochromators, receiver characteristics, electronic circuits for measuring the output of the receiver, characteristics of photographic plates, details of plate processing, and microdensitometry. Repetition of this material in such detail is not planned for this section. Instead emphasis will be placed upon commercially available spectrophotometers and secondarily on spectrophotometers of special design that have been described in the literature, because, it is thought, the major portion of the spectrophotometric work of the biologist can be satisfied by commercial instruments, with, perhaps, some minor modifications. The more adventuresome biologist will find at the end of this chapter the literature references dealing with details of each component of the spectrophotometer should he be confronted with a problem requiring the construction of a special spectrophotometric instrument. However, in this chapter it will be necessary to outline certain facts about each of the components of the spectrophotometer in order to make coherent subsequent discussion of complete instruments.

2. Components of a Spectrophotometer

All instruments for absorption spectroscopy or any spectroscopy for that matter are composed of the following elements: (1) a source of radiation, (2) a sample, (3) a device for isolating a portion of spectrum emitted by the source, (4) a suitable radiation detector, and (5) a device for measuring the response of the detector. The pertinent points concerning each of these components will now be considered.

a. Sources

Many of the standard references on spectroscopy have covered the general subject of sources in a very thorough manner. The reader is strongly urged to consult these references in order to become conversant with the terms employed in radiometry and with some of the properties of sources. For the most part commerical spectrophotometers have settled on one or two types of sources: (1) the thermal or incandescent sources for the near ultraviolet (4000–3200A) and (2) the hydrogen arc or discharge lamp for the remainder of the ultraviolet spectrum down to the beginning of the vacuum ultraviolet.

The reason for this is simple. Both of these sources emit what is known as a continuum, i.e., a spectrum in which all wave lengths in the useful portion of the spectrum are representey. Under such circumstances measurements of absorbance may be made at any point in the spectrum. It was the ready availability of convenient sources of continuous radiation which made possible the development of the commercial recording photoelectric spectrophotometers.

Laboratory spectrophotometers, particularly those instruments combining the microscope and the spectrophotometer, have made use of the radiation emitted by mercury or other metallic vapors excited by the passage of an electric current. Such sources have a more or less discontinuous spectrum.[4] For this reason most of the emitted energy is concentrated in a few narrow bands of radiation. This situation makes it easier to isolate narrow bands of radiation of relatively high energy content thus simplifying the apparatus for microspectroscopy considerably (Cole and Brackett, 1940). On the other hand, the wave lengths at which absorbancy can be measured are limited to those points at which there are lines of sufficient intensity (cf. Caspersson, 1936). The relative intensity of a number of commonly used sources including such recently developed ones as the Wyckoff-Jaco high intensity hydrogen discharge lamp (Finkelstein, 1950), the Nestor

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[4] The light emitted by metallic atoms should be emitted as discrete lines. The amount of continuous background depends on the operating temperature of the lamp and therefore on the vapor pressure of the emitting vapor.

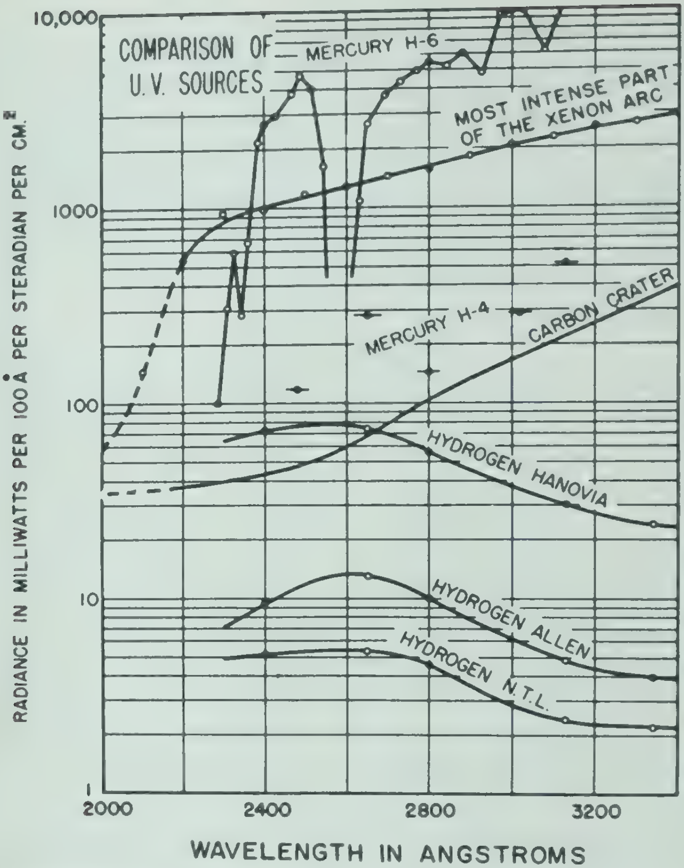


FIG. 27. Comparison of the spectral radiance of some ultraviolet light sources. Reproduced from Baum and Dunkelman (1950).

modification of the H_2 lamp designed by Allen (1941), and the xenon lamp (Baum and Dunkelman, 1950) are illustrated in Fig. 27.

It is important to bear in mind that there is a fixed relation between the size of a source, real or virtual, the size of an image of that source and the aperture of the image-forming system (LaGrange's law; see Hardy and Perrin, 1932, p. 43). This relation is illustrated in Fig. 28. The immediate

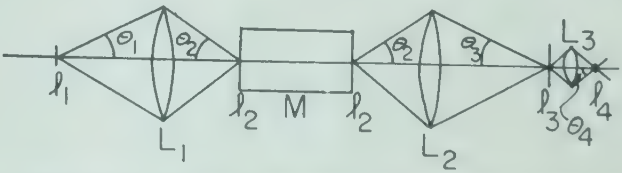


FIG. 28. $l_{1,2,3,4}$ represent a linear dimension of a source (l_1) and subsequent images thereof: $L_{1,2}$, field lenses; L_3 a microscope condenser; M a monochromator; $\theta_{1,2,3,4}$ half angles of the maximum cones of radiation.

$$l_1 \sin \theta_1 = l_2 \sin \theta_2 = l_3 \sin \theta_3 = l_4 \sin \theta_4$$

consequence is that when any one of the products (N.A. × l) becomes established either because of the geometry of the light source [luminous volumes such as gas discharge tubes present special problems (Wyckoff, 1952)], slit dimensions combined with monochromator aperture, or by experimental requirements, then the value for the same product for every other component is established. This fact is often of significance in the design of spectrophotometers in obtaining the maximum limit of requirements for expensive optical parts (Loofbourow, 1950; Blout, 1954).

b. Sample

The consideration of the sample, solvents, cells, and other pertinent data under this heading is best undertaken after the discussion of the components of the instrument.

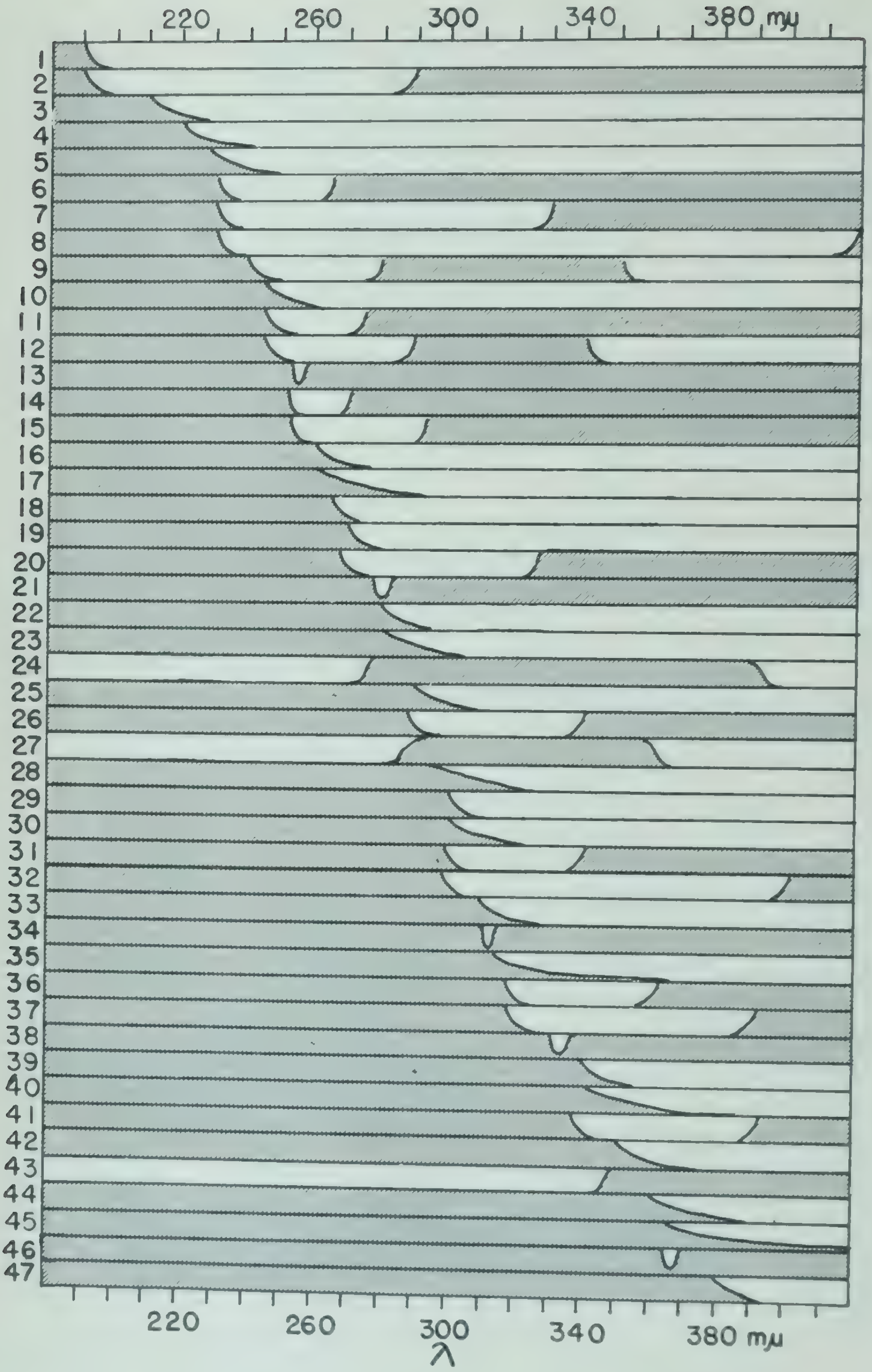
c. Special Isolation Component

In general the desired portion of the spectrum can be isolated by one or two means, and such isolation can take place before or after the sample. The unwanted portion of the spectrum may be absorbed and the desired portion transmitted as in a filter; or the radiation may be dispersed and the desired portion of the spectrum selected from the dispersed radiation on the basis of its position. The latter technique is employed in spectroscopes and monochromators, while the former method is employed in what is known as abbreviated spectrophotometry or colorimetry. Colorimetry has been but little used in the ultraviolet portion of the spectrum, but it appears that such instruments are feasible.

(*1*) *Filters.* Filters function by removing or rejecting some portions of the spectrum while permitting other portions to pass. This may be accomplished by absorption, interference, scattering, or rotary dispersion. Filters have the advantage of essentially unlimited aperture, low cost, and small physical dimensions compared with dispersive components. They have the disadvantage of being much less selective than dispersive components, much less flexible, and unstable in many instances. The latter disadvantage is much more serious in the absorption filters composed of solutions of organic compounds which in many cases must be renewed frequently.

Absorption filters: These are the most common type of filter used in the ultraviolet. They are of three general types: band-pass filters, filters designed for specific emission lines, and cut-off filters. Figure 29 gives the approximate position of the absorption and transmission bands of certain filters together with references to these filters. It should be pointed out that many solvents may be used as cut-off filters. Figure 35 of solvent absorption provides the spectral data for some solvents.

Interference filters: Interference filters are discussed in the section on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"Visible Absorption." The use of these filters in the ultraviolet is limited to the 3600–4000A because of the small choice of dielectrics and the low reflectivity of metal films in the ultraviolet.

Christiansen filters: This is a unique filter which depends upon selective scattering, has not been widely used in the ultraviolet. It has excellent potentialities for use in a colorimeter. The data for the construction of these filters and the optical conditions required for their use have been provided by the following papers: Kohn and von Fragstein (1932), Regner (1936), Minkoff and Gaydon (1946); and Sinsheimer and Loofbourow (1947).

(*2*) *Dispersive elements.* Spectrographic instruments and monochromators for the ultraviolet employ both prisms and gratings as the dispersing elements. The schematic outline of a monochromator is shown in Fig. 30. It will be seen that the middle element called "Dispersing Element" is not specified. This element can be a single dispersing element or two or more such elements can be employed in tandem. Rarely more than two are so employed. These instruments are known as single, double, and multiple monochromators respectively. Recent developments have made it possible to use the same dispersing element more than once, thus making a double or multiple monochromator from a single monochromator (Lee, 1950; Walsh, 1951, 1952).

There are in general at least five characteristic properties of a monochromator to be considered. They are dispersion, resolution, aperture, stray radiation, and transmission. These factors form the basis for judgment in the choice of a monochromator.

Prism: Quartz, either fused or crystalline, has been the principal material for the construction of prisms for the ultraviolet. Crystalline quartz has in

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FIG. 29. Filters for the ultraviolet. The unshaded areas represent the portion of the spectrum transmitted. The numbers to the left indicate the references listed below. 1–Haas (1935). 2–Heidt (1939). 3–Maclean *et al.* (1945). 4–Corning (1948). 5–Bass (1948). 6–Backstrom (1940). 7–Backstrom (1940). 8–Corning (1948). 9–Kasha (1948). 10–Bass (1948). 11–Kasha (1948). 12–Kasha (1948). 13–West (1949). 14–Bowen (1946). 15–Kasha (1948). 16–Kasha (1948). 17–Corning (1948). 18–Bass (1948); Maclean *et al.* (1945). 19–Bass (1948); Bowen (1946); Maclean *et al.* (1945). 20–Dorcas and Forbes (1927). 21–Backstrom (1940); Bucher and Kaspers (1946). 22–Bass (1948); Bowen (1946); Maclean *et al.* (1945). 23–Schott-Jena (1952); Corning (1948). 24–Gibson and Bayliss (1933); v. Halban and Siendentopf (1922). 25–Corning (1948). 26–Kasha (1948). 27–Bowen (1946). 28–Backstrom (1940). 29–Saunders (1928); Kasha (1948). 30–Corning (1948); Schott-Jena (1952). 31–Kasha (1948). 32–Corning (1948); Schott-Jena (1952). 33–Kasha (1948); Schott-Jena (1952). 34–Backstrom (1940); Bowen (1946); Bucher and Kaspers (1946); Hunt and Davis (1947); West (1949). 35–Maclean *et al.* (1945). 36–Kasha (1948). 37–Corning (1948). 38–Bowen (1946); Bucher and Kaspers (1946). 39–Kasha (1948). 40–Corning (1948). 41–Kasha (1948). 42–Schott-Jena (1952). 43–Acton *et al.* (1936). 44–Bowen (1946); Corning (1948). 45–Corning (1948). 46–Bowen (1946); Corning (1948); West (1949). 47–Eisenbrand and von Halban (1930).

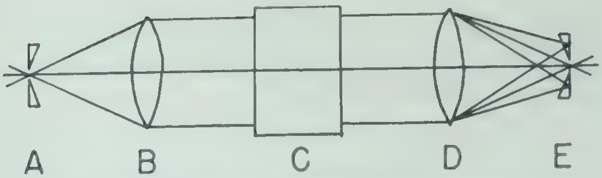

FIG. 30. Schematic diagram showing the optical elements common to all monochromators: A. Entrance slit. B. Collimating element. C. Dispersing element. D. Focusing element. E. Dispersed radiation at exit slit.

general better transmission in the short wave length portion of the ultraviolet, but even crystalline quartz varies greatly from specimen to specimen in transmission around 1900A. A variety of types of mountings are available and standard methods for coping with the birefringent properties of the crystalline quartz are employed.

In special instances where monochromators of high aperture have been required use has been made of hollow quartz prisms which are filled with water as the principal dispersing medium to avoid the excessive cost of large prisms of quartz. Prisms with faces 10 inches in diameter have been constructed in this fashion (Fluke and Setlow, 1954; Harrison, 1934; Forsythe, 1937; Cannon and Rice, 1942).

Gratings: Grating monochromators are now commercially available and the best spectrographic instruments have for many years employed gratings as the dispersing element. In the past grating instruments used plane gratings ruled on a transparant support. These are still used in some commercial spectrophotometers limited to the range above 3500A. Recent developments have permitted the production of high quality reflecting gratings ruled on a concave surface at a price which is competitive with prism instruments. The grating monochromators for the ultraviolet are of two types: a modified Wadsworth-mounted concave grating (Bausch and Lomb), and a Littrow-mounted plane reflecting grating (Perkin-Elmer). The former grating is ruled in such a way as to concentrate 63 % of the incident energy of a given band width into that band in the first order spectrum. Both monochromators employ reflecting optics throughout and hence are essentially achromatic over the entire spectral range.

Grating monochromators have an advantage in that the dispersion is linear and thus the complicated drive cams or dispersion curves required for prisms are not needed. The average dispersion of the gratings commercially available is generally greater than that of prisms. In general, the stray radiation from a grating monochromator tends to be lower than that from a prism monochromator although aging of the reflecting surfaces of the grating and other mirrors produces an increasing amount of stray radiation. Aging of a mirror also leads to a sharp decline in the reflectivity especially at shorter wave lengths. This is also true of prism monochromators employing mirrors. The grating monochromator is somewhat more delicate and the grating itself must be treated with great care and kept scrupulously free of dust.

The preceding discussion would suggest that a carefully maintained grating monochromator employing the double pass system of Walsh (1951, 1952) with the added refinement of a small predispersing prism would yield an instrument combining the advantage of both prism and grating instruments and eliminating many of the disadvantages inherent in either.

d. Detectors

(*1*) *Fluorescent ultraviolet.* Fluorescent screens have been used in ultraviolet spectroscopes and for focusing and alignment work with ultraviolet optics. Fluorescent materials have been combined with photoelectric detectors to extend the range of the photoelectric detector (Johnson *et al.*, 1951; Dejardin and Schwegler, 1934). In this case the fluorescent material acts as a converter by absorbing the ultraviolet that would not penetrate the phototube jacket and reradiating the energy at a longer wave length, which will pass through the jacket.

(*2*) *Chemical.* Chemical detectors include photographic materials and actinometers. No attempt will be made to discuss either in this chapter. The reader is referred to Harrison *et al.*, (1948, Chap. 13), Brackett and Forbes (1933), and to Calvert and Rechen (1952).

(*3*) *Thermal.* Thermal detectors are but little used in the ultraviolet. Included in this category are thermocouples, thermistors, bolometers, and pneumatic detectors. The main feature of these detectors is that all wave lengths of the optical spectrum are completely absorbed, converted into heat, and the temperature rise of the detector is determined from its response. If the heat capacity of the detector is known then the amount of energy absorbed can be calculated. These detectors are often used for absolute energy measurements, for calibrating other detectors and sources.

(*4*) *Photoelectric.* Electric detectors depend upon the fact that the absorption of light by certain materials can yield enough energy to free one or more electrons by removing them from the atoms of the active material.[5] Detectors of this type fall into three classes: photovoltaic (barrier layer) cells, photoemissive cells, and photoconductive cells. In the photovoltaic cell a potential difference appears across the terminals of the cell when it is illuminated, which can be used to produce a current in an external circuit. When quanta of sufficient energy are absorbed by the sensitive surface of a photoemissive cell electrons are ejected from that surface into adjacent free space. A potential difference is imposed between the sensitive cathode

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[5] A discussion of the active materials of photocells for the ultraviolet is found in Zworykin and Ramberg (1949).

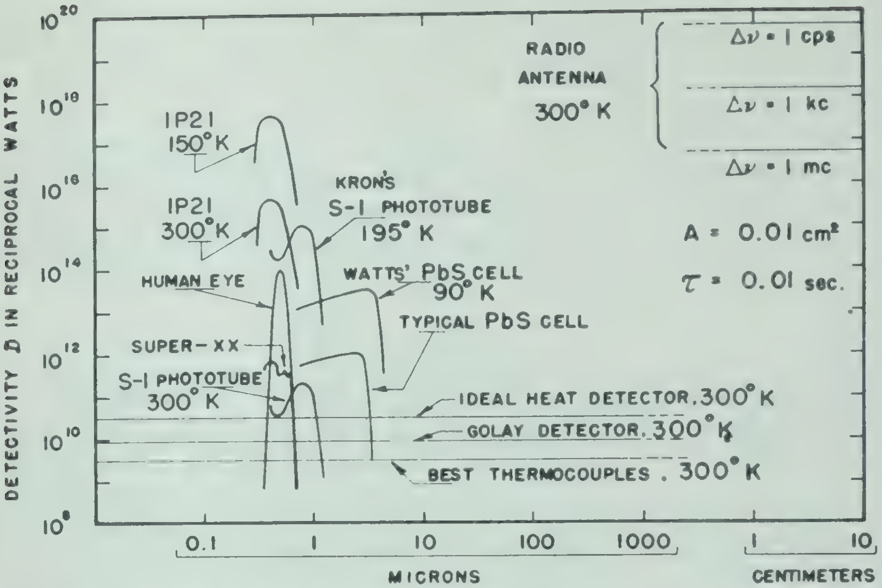

FIG. 31. Detectivity plotted as a function of wavelength for a number of different kinds of detectors. Reproduced from Jones (1953).

surface and the anode. Under the influence of this field the free electrons move from the cathode to the anode and into an external circuit. Quanta absorbed by a photoconductive substance release electrons into the solid through which the electrons are then free to move, causing an apparent decrease in the resistance of the detector.

A modification of the photoemissive cell is the photomultiplier, more properly called an electron multiplier. In this detector electrons released from the photocathode are attracted by a potential difference to a second sensitive surface. For each electron striking the second surface two or more electrons are released. These in turn are attracted to a third surface, etc., until the electrons from the last surface are collected at the anode. Amplifications of from 10^5–10^7 may be obtained in such tubes.

The over-all response of a number of detectors is depicted in Fig. 31, taken from a recent paper by Jones (1953).

e. Measuring Devices

A spectroscope, spectrograph, or spectrophotometer requires, in addition to the detector, some device for translating the primary response of the detector into some easily measurable quantity. Some commonly employed devices are briefly discussed below.

(*1*) *Eye.* The eye is one of the most sensitive detectors of visible radiation and is capable of discerning very small gradients of density over a tremendous range of brightness. This quality of the eye makes it a valuable adjunct in looking for small slight inflections or very small maxima, which are hard to distinguish in spectrographic plates behind the electrical and grain noise of a microdesitometer trace of the same plate. The eye is thus primarily a qualitative detector-indicating combination.

(*2*) *Densitometers.* A microdensitometer is an instrument for measuring the degree of blackening of a small area of a photographic plate. A small spot of light is imaged on the spectrographic plate and the light transmitted by the plate is collected by a microscope objective and projected onto an adjustable slit behind which is a detector, either thermal or photoelectric. The output of the detector is then recorded either point-by-point along the length of the spectrographic plate or automatically as the plate is translated continuously by a very precise drive mechanism. The result is a graph of plate blackening *vs.* wave length. If the plate has been calibrated with regard to sensitivity for the spectral range used then plate blackening can be translated into absorbance terms (Harrison *et al.*, 1948, Chap. 13).

(*3*) *Photometers.* Thermal and photoelectric detectors respond to light by the development of a potential difference across the detector or by a change in the resistance of the detector. Detectors of this type have been used widely and a great variety of circuits for photometry based upon them have been devised and described in the literature (Zworykin and Ramberg, 1949). Many are quite simple while others require complex amplification and servo mechanisms. Despite the diversity of such circuits they can be categorized as either direct reading or null instruments either of which may be adapted for manual or automatic recording operation.

Direct reading instruments are characterized by the fact that the deflection of the indicating device is a measure of the response of the detector. Many photometers measure the photocurrent directly or after amplification by means of a sensitive galvanometer, a microammeter, or a milliammeter. Recently there has appeared on the market a very sensitive and relatively rugged microammeter which responds rapidly and accurately down to 0.01 μA which should prove quite useful in laboratory-built photometers.[6]

In some direct-reading photometer circuits the photocurrent is passed through a fixed resistance and the potential developed across the resistance is measured with a vacuum voltmeter.

Any direct reading instrument can be converted into an automatic recording instrument by using a meter which records as well as indicates the output of the photodetector.

There are many commercial examples of direct reading instruments: The Coleman spectrophotometer and the Evelyn colorimeter are examples of the manual type, and the Perkin-Elmer Model 12 infrared spectrophotometer is a good example of a direct recording photometer.

The null circuit differs fundamentally from the direct reading type. In the former circuits and accurately variable potential derived from an external source is adjusted for each reading of intensity in such a manner that the variable potential is equal to and of opposite sign to that developed by the receiver. This is known as an electrical null. An alternative procedure, known as the optical null, provides a means for varying the intensity of the light falling on the detector in an accurately known manner. In either method the setting of the attenuation device, which yields a given position of the indicating meter, is taken as proportional to the intensity of the light falling on the detector. By either the electrical or the optical null method a device can be had that will indicate the position of the attenuator and thus make the instrument an automatic recording photometer.

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[6] R.C.A.

With both the direct reading and the electrical null method the assumption is made that the response of the photocell is a linear function of the incident light intensity (see page 192). The direct reading instrument also involves linearity of the indicating meter. In the electrical null method a relatively simple meter of very limited range and high sensitivity may be used. Here the burden of linearity has been transferred from the meter to the potentiometer. In instruments involving amplification of the primary detector response the amplifiers must be linear or logarithmic. Modern techniques of production have made potentiometers linear or logarithmic to 0.1 % available at reasonable cost. In the optical null method the burden of linearity has been transferred from the detector, amplifier, meter, and potentiometer, and placed on the device used to attenuate the intensity of the light beam.

Considering the wide use of photoelectric detectors it might be worthwhile to consider briefly some of their fundamental characteristics. One of these characteristics is sensitivity, which is the slope of the curve relating the photocurrent produced to the intensity of radiant energy incident on a given area of photosensitive surface. Sensitivity will vary with wave length in all phototubes, and it frequently varies with the voltage applied to the tube. In all photoelectric detectors there is some random emission of electrons even in the complete absence of light. The randomly fluctuating current produced by these electrons is known as the dark-current. The magnitude of the dark-current decreases exponentially with the temperature of the sensitive surface. It can be demonstrated that the accuracy of measuring the photocurrent is related to the relative magnitude of the photocurrent and the mean value of the random fluctuation of the dark-current. This is known generally as the signal-to-noise ratio.

Another important characteristic of a photocell is the threshold, which may be defined as that intensity of light that will produce a photocurrent just equal to the mean value of the random fluctuations of the dark-current.

Now consider what happens to these factors on amplification. The sensitivity is multiplied by the amplification factor. Such amplification, while elevating the photocurrent, will also elevate the dark-current by exactly the same factor. Thus amplification increases the sensitivity without changing the threshold of a photocell. The accuracy of measurement of a weak signal which is near the noise level is not increased fundamentally by any process which equally amplifies both the dark-current and the photocurrent.

This point is frequently overlooked in connection with the use of the photomultiplier tube. The relatively large electrical signal from these tubes for very low light levels is looked upon as enabling one to measure the weaker photocurrents (further into the ultraviolet) with greater accuracy. In fact the threshold of the photomultiplier is essentially the threshold of the photocathode and the accuracy of measurement of weak currents has not been greatly altered.[7]

One way in which the signal-to-noise ratio can be improved depends upon the fact that the random omission (noise) is spread widely over the frequency spectrum. If the photocurrent selected is an alternating current of a known frequency, then only a small portion of the total dark-current has this frequency. If the amplifier for the phototube output is so constructed that it passes only a narrow band of electrical frequencies centered on the frequency of the photocurrent, then essentially all of the photocurrent passes the amplifier but only that part of the noise which lies within the band pass of the amplifier will be amplified. This results in a large gain in the signal-to-noise ratio. This property, together with the high stability achievable by feed-back, is the principal argument in favor of a-c amplifiers in photometry. The improvement in the signal-to-noise ratio achieved by the use of tuned a-c circuits introduces a problem concerned with the speed of response of the amplifier (the time required for a certain percentage of the true value of an instantaneous change in input to appear in the output of an amplifier). In general the reduction in noise goes down as $1/\sqrt{\text{bandwidth of amplifier filter}}$ while the response time goes up linearly with 1/bandwidth of amplifier filter. For this reason tuned a-c amplifiers are a compromise between reasonably rapid response and high signal-to-noise ratio.

Direct current amplifiers are usually somewhat simpler in design, but have problems of zero drift that are not easily solved. Certain spectro-

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[7] The phototube is not the only source of noise in the whole circuit. Noise also arises in the load resistance and in the first stage of the amplifier. Since the photomultiplier produces a larger signal for a given light intensity a lower value for the load resistance can be used thus reducing the resistance component of the over-all noise.

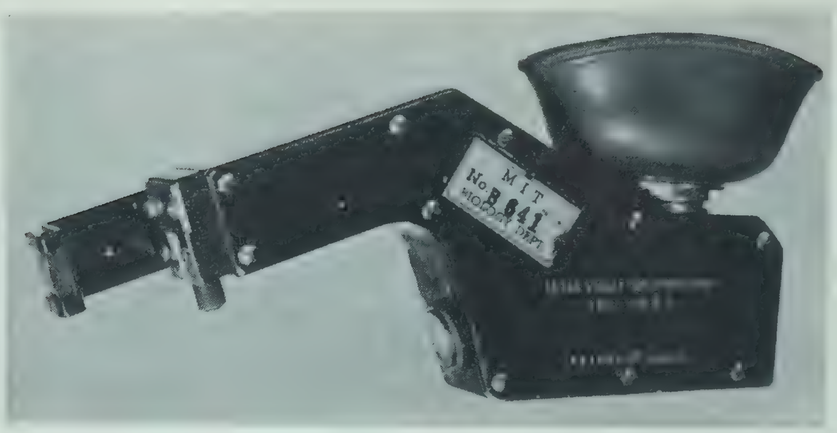

FIG. 32

photometer circuits make use of d-c circuits. Notable among these is the Beckman DU spectrophotometer.

It is impossible to go into the details of the fundamental nature of the signal-to-noise ratio in measurements and into the details of the advantages and disadvantages of various d-c and a-c amplifier circuits in this chapter. This is, however, an important matter, especially for anyone concerned with the measurement of very low light levels such as are met in ultraviolet microspectrophotometry. The interested reader will find these topics covered adequately in the references at the end of this chapter.

3. SPECTROSCOPES

A hand spectroscope for the ultraviolet is illustrated in Fig. 32. This instrument is made by Beck & Company of England. It has an adjustable entrance slit through which radiation passes to a small quartz lens and then through a small quartz prism. The dispersed radiation is focused onto a fluorescent screen which is observed by eye with the aid of an ocular fitted with a rubber eyecup to exclude surrounding light. There is an adjustable scale adjacent to the screen which is illuminated with white light through the small window below the slit. The accuracy of this instrument is relatively low but it has great utility when one wishes to make semiquantitative observations such as the wave length of cut off of solvents, dielectrics, the general position and relative intensity of certain emission lines of sources, and the degree of contamination of hydrogen lamps with other substances such as hydrides and cathode coating material. We have found this instrument of particular value in the rapid sorting of quartz, Vycor, Corex and other glass which are inevitably mixed in the laboratory. This is particularly true of microscope slides and cover glasses.

4. Spectrographs and Photographic Spectrophotometers

The grating and quartz prism spectrographs formed the backbone of the earlier spectroscopic work in the ultraviolet, and more recently have been employed for special work by biologists when no readily available photoelectric instrument would suffice. Spectrographs have in general higher resolution than most of the available spectrophotometers, but the procedures for photographic spectrophotometry are very time consuming. In the author's experience (Scott *et al.*, 1948) 16 hours were required to produce a plot of absorbancy *vs.* wave length by the photographic method of Henri (1913). The same information can now be obtained in 20 man minutes with an automatic recording photoelectric spectrophotometer (Cary).

An adaptation of photographic photometry of interest to the biologist has been devised by Holiday (1937). It is in essence a modification of the method of Henri. Instead of increasing the exposure time discontinuously as Henri proposed, Holiday devised a motor-driven cam that moves the photographic plate upward in the focal plane in such a way that the image of the spectrum, which occupies but a narrow strip of the plate at any moment, is allowed to fall on any given portion of the plate for a period of time that decreased continuously, logarithmically, and reproducibly. The results of this technique are seen in Fig. 33. The contour of the absorption curve is easily visualized in great detail. The height above the base line of a given degree of plate blackening for a given wave length may be taken as proportional to the absorbance of the sample at that wave length. To produce a spectrum in similar detail, when such detail is present, would certainly require a great deal of labor with a point-by-point machine if it was possible at all. The fine structure would not be as strikingly defined in the point-by-point instrument as it is in the moving plate due primarily to the extreme sensitivity of the eye to small changes in density. There is some question, however, whether for routine biological work an automatic recording spectrophotometer is not capable of yielding the same information in a much shorter period of time.

Photographic absorption spectrophotometry is so little used today and the subject has been covered in detail in texts on spectroscopy that it will not be necessary to consider this method further in this chapter.

5. Photoelectric Spectrophotometers

a. Manual.

Among commercial spectrophotometers the Beckman DU and its English counterpart, Unicam, can be credited with making ultraviolet absorption spectrophotometry as easy to perform technically as visible spectropho-

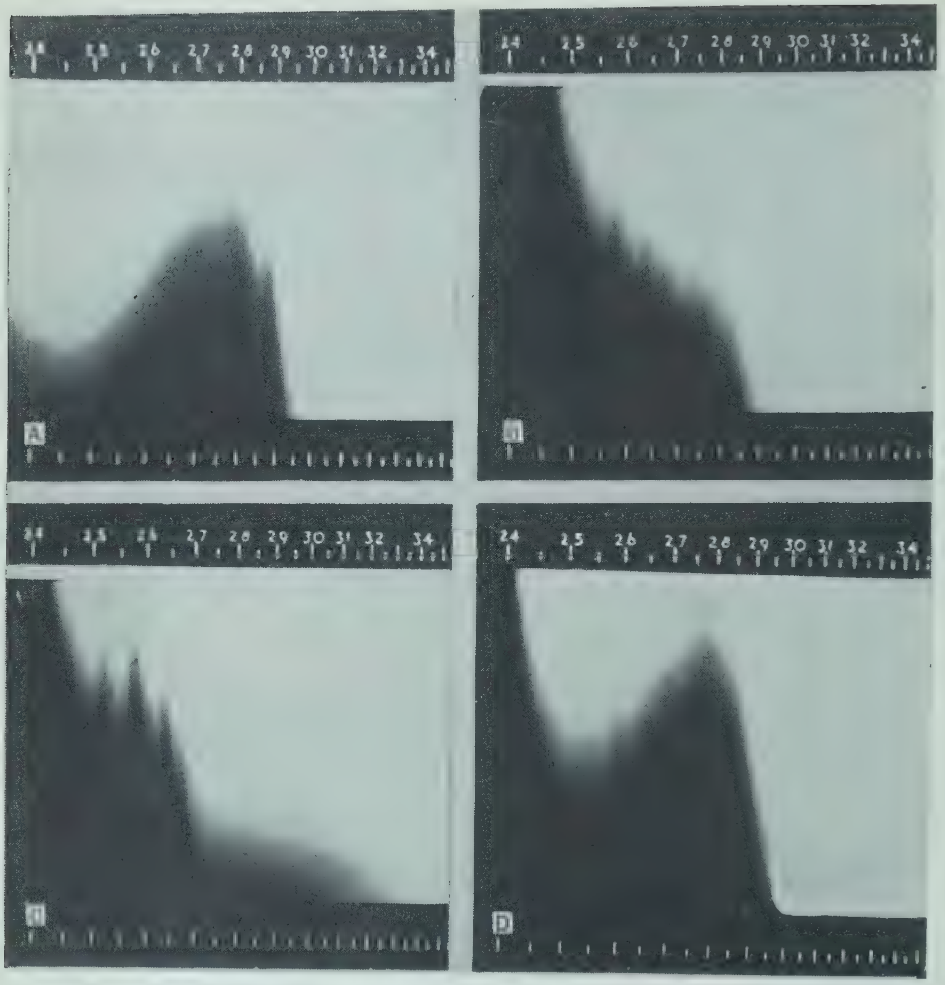

FIG. 33. Moving-plate spectrograms of polypeptides and proteins. A. Gramicidin in EtOH. B. Gelatin sol. C. Polymixin. D. Bovine serum albumin. Wavelength scales of spectrograms are in A. × 10^{-7}. From Beaven and Holiday (1952).

tometry, thus placing this powerful tool easily within reach of the biologist. Indeed the Beckman spectrophotometer has become an almost indispensable tool in research laboratories in which any analytical work is done. For these reasons the Beckman DU will be described in some detail.

The Beckman employs two light sources, both producing a continuous spectrum in the range recommended for their use. The tungsten filament emits a usable quantity of energy down to about 3500A. The tungsten source must be used with caution in this region because of the large stray light effect (see below). A filter is furnished with the instrument and should always be used in conjunction with the visible source in the near ultraviolet. The source for the remainder of the useful ultraviolet spectrum is a hydrogen lamp driven from a constant voltage source. A concave mirror focuses the source on the entrance slit. This mirror is often the source of difficulty as discussed below.

The entrance and exit slits are one above the other, the entrance slit being the lower. They are controlled synchronously and symmetrically by the knob on top of the instrument, the physical width of the slits being indicated by the dial below the control knob.

The entrant beam is collimated by a first surface spherical mirror and directed onto a 30° fused quartz prism, the back face of which has a reflecting coating. The dispersed radiation is focused on the exit slit by the same mirror used for collimation. The nominal wave length of the band of radiation emerging from the monochromator is determined by the angular setting of the prism which is in turn controlled by a knob from the top of the instrument. Below the knob is a scroll dial from which the approximate wave length is read. The beam emerging from the monochromator passes through the sample compartment and enters the phototube compartment through a shuttered aperture. There are two phototubes in the regular DU, both photoemissive. One of these is primarily sensitive to long wave length visible radiation and the other, the "blue sensitive" tube, is used from 6250 A throughout the ultraviolet. The phototube compartment also contains a load resistor, and electrometer tube, and a single stage d-c amplifier.

The monochromator and phototube compartment of the instrument are provided with containers of silica gel to reduce the relative humidity within these compartments. Under conditions of high relative humidity it is very important to regenerate or renew the silica gel frequently to avoid erratic operation of the instrument and poor reproducibility. It is recommended that tags be attached to the appropriate compartments on which can be noted the dates of renewal of silica gel, of battery replacements, etc.

The Beckman instrument is provided with a potentiometer for cancelling the dark-current of the phototube. It has been found advisable to determine the precision of balance of the dark-current knob both before and after each reading of absorbancy.

The various portions of the Beckman spectrophotometer should be considered in some detail since this will serve to illustrate certain points common to all ultraviolet photoelectric spectrophotometry.

The light sources furnished are reasonably adequate for the ranges usually employed. Other sources have been employed with essentially the same optics except that a plane mirror has replaced the usual light source. The auxiliary source is focused on the plane mirror which then becomes a virtual source from which light is directed into the monochromator by the standard optics. In this manner the AH-6 high pressure mercury arc (GE) has been employed (Commoner, 1948) as well as hydrogen sources such as that manufactured by Nestor (Allen, 1941) and employed in the Cary spectrophotometer. A similar arrangement could be made for the newer, more intense, Wyckoff-Jaco hydrogen lamp (Finkelstein, 1950), or the high intensity xenon source (Baum and Dunkelman, 1950). The use of these sources might well extend the useful range of the Beckman to the limit of its wave length cam (2000 A).

The optical adjustment of the Beckman is accomplished by the movement of light source, the condensing mirror, and the 45° plane mirror which directs the light into the monochromator. The gross adjustment of alignment is best accomplished by removing the phototube compartment and applying one's eye to the exit slit. The various adjustment screws are then manipulated until the collimator, which is clearly visible through a wide slit, is seen to be illuminated with maximum uniformity and brilliance. The phototube compartment is then replaced and, using the electicral circuit to measure energy, and final adjustment is made to maximize the energy reaching the photocell (minimum slit for a given wave length and sensitivity setting). The adjustment by eye is usually extremely close to the electrical maximum.

Mirror optics are used throughout with the exception of the prism and a lens which covers the exit window of the monochromator. As noted earlier, first surface mirrors required for work in the ultraviolet have a tendency to age rapidly. The short wave-length limit of the Beckman will change with time due primarily to the change in the mirrors, although also the light source will decline in intensity. In addition to the change in the short wave-length limit, there is also an increase in the stray radiation with time (see below).

Since the Littrow prism is operated from a cam, it is of course unnecessary to point out that the indicated wave lengths on the scroll dial are only approximate. It will be necessary for each instrument to be calibrated as noted below. Over a number of years the cam of the wave length drive mechanism may become worn and it may be wise to recheck the wave length calibration annually.

The potentiometer knob carries two scales. One of these is linear, indicating that the potentiometer is itself linear and reads %T (transmittance), while the other scale is logarithmic and is "Optical Density" (absorbancy). These scales must be calibrated as noted below.

The circuit of this instrument operates on the electrical null principle. The null indicator is the meter mounted on the top surface of the instrument. The spectrophotometer may be operated in a number of ways. The most common operating procedures involve fixing the slit width for a given band width by reference to the data furnished with the instrument and adjusting the 100% T (O A) signal level with the "Sensitivity Knob." This sensitivity knob controls a potentiometer which regulates the voltage spanned by the absorbancy potentiometer. It will be seen that a change in setting of this knob changes the increment of voltage per turn of the absorbancy potentiometer as well at the voltage corresponding the 100% T. The latter affects the 100% T balance and the former affects the amount of movement of the null indicator meter for a given amount of rotation of the absorbancy dial. This latter fact is of some importance, as will be noted in Chapter 3 under the heading of errors in spectrophotometry. An alternative method of operation is to fix the setting of the sensitivity knob, and to balance for 100% T by control of the light intensity by change in the slit setting. If the sensitivity knob is positioned to yield the minimum slit width for a given wave length, this technique then gives spectra of maximum resolution but of lowest accuracy with regard to absorbance measurements.[8]

It was mentioned earlier in this section that many of the instruments for use primarily in the visible portion of the spectrum may also be used below 4000 A to the limit set by the combination of glass optics and the operating temperature of the tungsten light source. Since the section on spectrophotometry in the visible (Chapter 3) has covered these instruments, no further comment will be made concerning their use in the ultraviolet except to note that the comments concerning the effect of stray light at a short wave length limit of the quarts spectrophotometer (see p. 190) apply with equal force in this case, and indeed to the case of any spectrophotometer which is operated near the limits of its wave length range.

Prior to the appearance of the Beckman spectrophotometer on the market, a large number of point-by-point ultraviolet spectrophotometers had been described in the literature (see for example, Harrison *et al.* 1948). The need for the construction of instruments of this sort in the laboratory has largely vanished.

A combination of the light sources, monochromator, detector, and indicating circuit has been used in conjunction with the microscope to permit the determination of an absorption spectrum on very small volumes of solution or small solids such as crystals or tissue sections. A sketch of one of these instruments is shown in Fig. 34. It is clear (Loofbourow, 1950;

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[8] The definition of sensitivity as applied to the Beckman is somewhat ambiguous and is in no way to be related to the term sensitivity used in the discussion of phototubes above. In the Beckman literature, decreasing sensitivity is used to mean decreasing deflection of the null indicating meter for a given rotation of the absorbancy potentiometer knob. The minimum sensitivity setting produces the minimum voltage drop across the absorbancy potentiometer and the minimum voltage appears as 100% transmission, thus requiring narrower slits to furnish the intensity to balance the smaller voltage equivalent to 100% T.

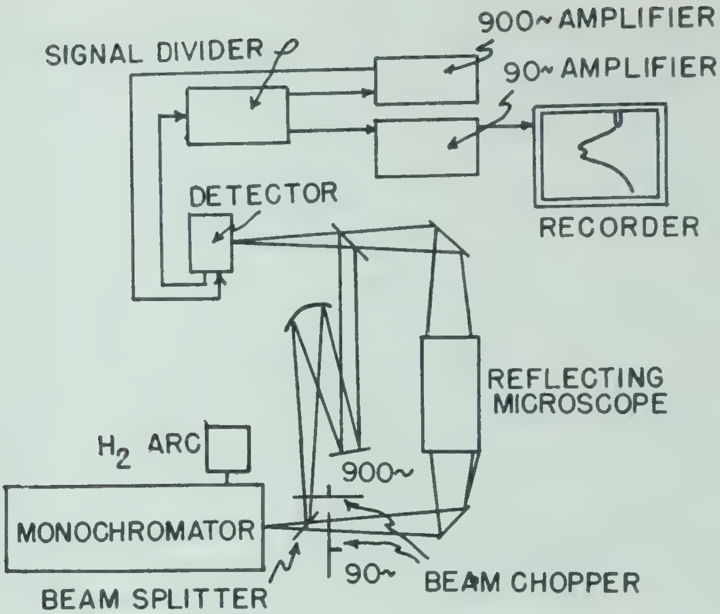

FIG. 34. Schematic diagram of a compensating automatic recording microspectrophotometer designed and built at the Massachusetts Institute of Technology. Redrawn from Wyckoff (1952).

Blout, 1953) that unless a very intense source of radiation is used a rather large band of radiation must be passed by the monochromator and consequently resolution may be quite poor. For such purposes high resolution is frequently not needed. Moreover the microsceptrophotometer is most often used as an abbreviated spectrophotometer or colorimeter and is calibrated for each analysis for the optical conditions which obtain. Another form of this instrument has been used by Norberg (1942) to measure total phosphorus of tissue in amounts as small as 10^{-10} g. More recently the combination of the microscope and photoelectric spectrophotometer has been utilized to measure the absorption of narrow paper strips of chromatograms to quantitate extremely small amounts of solute contained thereon. Edstrom (1953) has used a similar technique to measure the amount of RNA in a single nerve cell.

Special problems associated with the combination of the microscope and the spectrophotometer have been considered in a number of places recently and are considered later in these volumes.

The Jarrell-Ash Company has developed a microspectrophotometer on the basis of the experience of a number of workers with the capillary tubing type of microcells described by Krugelis (1950) and by Malmstrom and Glick (1951). This versatile instrument should be a considerable improvement over the instruments which combine the microscope with the spectrophotometer for microspectrophotometric analysis. As designed it can be connected to a ratio recorder for automatic operation. (Glick and Brech, 1954).

b. Automatic Spectrophotometers

Beckman has produced a machine (Beckman, 1953) designed around the DU spectrophotometer which automatically goes through the whole series of adjustments made by the operator of the manually operated DU and makes an ink mark on a piece of special graph paper to indicate the transmittance at that wave length, advances to the next wave length, and repeats the operations. It will handle up to three samples in one run, printing the transmittance for each sample in a different color. This instrument is relatively slow in its operation and has little if any advantage over the continuous recording spectrophotometers described below.

Cary (1947) designed and produced one of the first automatic recording spectrophotometers for both the visible and ultraviolet spectrum. This instrument employs the hydrogen discharge tube designed by Allen (1941) and manufactured by Nestor,[9] and a tungsten filament bulb as sources of radiation for the useful spectral range. The radiation is dispersed by means of a double monochromator for employing Littrow mounted fused quartz prisms. The wave length scale is made linear with time (chart length) by means of a cam. The performance of the monochromator is excellent. The author's instrument has less than 0.3 % scattered light of λ greater than 2300 A at all monochromator settings less than 2300 A. Emission lines separated by 1.5–2.0 A are readily resolved throughout the ultraviolet and it has been reported that the Hg lines 2482.72 and 2482.00 are resolved by the monochromator (Heidt and Bosley, 1953). The monochromatic beam is split into two beams in an aperture plane by a multiple first surface prismatic mirror. One of the beams emerges through one exit slit and traverses the sample compartment while the other beam emerges through a second exit slit and traverses the control compartment. The cross sectional shape of the beams within the compartments is controlled by lenses placed immediately after the exit slits.

The electronic portion of the instrument employs two photomultipliers as detectors, one for each beam. The differences in the response *vs.* wave length curve for the two photocells are empirically corrected by an ingenious multiple potentiometer arrangement. The slit width is controlled automatically in such a way that the signal generated in the control beam photocell is maintained at a predetermined level, which is adjustable. The instrument in operation compares the magnitude of the signal from the control and from the sample phototube. When there is a difference between these signals the pen of a continuous strip chart recorder is caused to move. As the pen moves the amplification in the sample circuit amplifier is altered to maintain constant deflection sensitivity. The movement of the pen can be linear (transmittance), logarithmic (absorbance), or log-log (log absorbance) depending upon the choice of slide wires for the recorder.

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[9] 152 W. Plumstead Ave., Lansdowne, Pa.

As the instrument is supplied, absorption cells up to 10 cm can be accommodated. An attachment is available which alters the geometry of the beam in the sample compartment in such a way that a cell 5 mm ID with a 1 cm path may be used (the minimum volume of this absorption cell is 0.2 ml).

Recently there have appeared two continuous recording spectrophotometers both of which utilize the Beckman monochromator. One of these is made by Beckman and the other by Warren Electronics.[10] The Warren instrument contains a "chopper" driven by a synchronous motor which interrupts the exit beam from the monochromator at 30 cps. The beam is then split by a prismatic device similar to that used in the Cary instrument and directed through a sample and control compartment. The slit width is controlled by a constant energy servo system much like the Cary. The receivers are photomultipliers which have been specially selected. The sample and control a-c amplifiers are interchangeable plug-in type units. A unique arrangement permits the instrument to operate at low transmittances without a decrease in the signal to noise level in the servo system. The over-all differences in the sample and control signals for 100% transmittance is compensated by manual adjustment of "trimmers." The instrument is furnished with a transmittance scale, but it can be obtained with an absorbance scale. Other accessories permit the recording of transmittance or absorbance as a function of time, and automatic repetitive scanning of any portion of the spectrum. The cell compartment will accept cylindrical cells up to 10 cm in length and the regular 1-cm square cells usually employed with the Beckman can be used as well.

There are no data in the literature on the performance of this instrument. The makers claim a precision of·0.2% and an accuracy of 0.5% for the photometer, without stating the absorbance range over which these figures apply.

Comments on the Beckman DU monochromator apply as well to this spectrophotometer.

Beckman has recently produced a continuous recording double beam spectrophotometer which uses a slightly modified DU monochromator. This is a versatile instrument in which the long wave length limit of the DU monochromator has been extended to 2.7 μ in the near infrared. Any portion of the transmittance scale down to 10% can be spread over the full chart width, a useful feature for revealing the details of absorption maxima. (Kaye *et al.*, 1951; Kaye and Devaney, 1952; Beckman, 1954).

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[10] Bound Brook, New Jersey.

6. ABSORPTION CELLS

Absorption cells of older design have been discussed in detail in standard textbooks on absorption spectrophotometry and will not be covered in this section. A wide range of absorption cells for special purposes are now available. The main differences in these cells is in the volume of solution required to give an unobstructed path for the light beam and the physical length of the light path.

a. Short Path, Small Volume Cells

Cells of this sort have not been produced commercially but have been described in the literature. In theory such cells can be made to contain extremely small volumes. Such cells would be particularly valuable when only simple chemical manipulations are carried out and when only very small amounts of material are available (Loofbourow, 1950; Blout *et al.*, 1952; Norberg, 1942).

b. Short Path, Moderate Volume Cells

Pyrocell[11] makes optical quality rectangular plugs of quartz which will fit into the regular Beckman quartz cuvettes. These are of known thickness and are furnished in pairs. The thickness of quartz effectively subtracts from the known path length of the regular cuvettes. Each plug affords two path lengths, since it is cut rectangular and can be inserted in either direction with respect to the beam. These are valuable adjuncts to spectroscopy, and are not as expensive as the variable path length micrometer cells (see below). Care must be exercised in calibrating these plugs especially at wave lengths less than 2,500 A. Below this wave length, differences in the optical quality of quartz may become quite large. With these plugs path lengths of 0.17, 2.0, and 5.0 mm can be obtained with regular Beckman cells of 10.00 mm path.

c. Long Path, Small Volume

Laboratory versions of the long path, small volume cells have been made of capillary tubing with temporary windows attached at each end for each determination (Krugelis, 1950; Malmstrom and Glick, 1951). These cells usually contain 5–10 ml in paths of 10–20 mm. Craig *et al.* (1953) have recently described a similar capillary cuvette for use with the Beckman which is commercially available.[12]

[11] Pyrocell Manufacturing Co., 207 E. 84th St., New York 28, N. Y.

[12] Microchemical Specialties Co., Berkeley, California.

d. Long Path, Moderate Volume

Vallee (1953) has described long path cells for use with the Beckman so designed as to include a full exit beam with a path of 5 cm. This cell requires a volume of about 6 ml. These cells have a definite advantage when, for technical reasons, reactions cannot be reduced in scale to use other type cells, or when one has limited amounts of the substance of limited solubility. The gain over the regular Beckman cuvette which requires 3 ml. for a 1 cm. path is $\frac{3}{6} \times \frac{5}{1} = 2.5$.

e. 1 cm. Path, Small Volume

Lowry and Bessey (1946) have described a simple modification of the Beckman DU which permitted the use of special cells containing minimum volumes of 100, 50, and 20 μl. This is achieved by placing a circular beam stop outside the exit slit of the monochromator and reducing the width of the cuvette until the sides of the cuvette are just clear of the beam. The small cylindrical beam is passed through the cuvette so that it just misses the bottom of the cuvette. The cuvette is then filled until the bottom of the miniscus of the free surface is just above the beam. The small stop after the exit slit reduces considerably the amount of energy and thus increases the short-wave limit of usefulness of the Beckman and/or decreases the absorbance of a blank against which the instrument can be balanced. For a given wave length the slits must be opened as the smaller stops are used. This of course will influence the absorbancy reading at that wave length. This apparatus is also extremely sensitive to misalignment since the path of the beam is so close to the walls and to the free surface. Because of this and the increased slit width employed, great care must be taken to insure that the equipment yields the correct result for some absorbancy standard (*e. g.* potassium chromate solution) within the error of the instrument. Verification of alignment is routinely done at the beginning of each day's work in this laboratory, since the instrument is also used with conventional cells. Somewhat more care is required in cleaning these cells because, with the small cross-sectional area of the beam, even small particles occlude a detectable amount of light.

A similar design is used by Glick and Brech (1954) for a microcell to be used in conjunction with the microspectrophotometer described above.

f. Variable Path Length Cells

The earliest absorption spectra were obtained with cuvettes of variable path length and absorbancy was expressed in terms of path length necessary to yield a fixed intensity of a transmitted monochromatic beam. Modern versions of the variable path length cells have been made by

Adam Hilger and by Perkin-Elmer. The latter micrometer cell is designed primarily for use in the infrared but can, by changing the windows, be adapted for ultraviolet work. These cells are particularly useful for measurements of the complete spectra at a single concentration since by adjustment of the path length all portions of the spectra, maxima and minima, can be brought into the range of optimum absorbancy. The precision and accuracy of these variable path length cells have been carefully considered by Hirt and King (1952). From their results it would appear that the precision and accuracy of the micrometer adjustment is relatively poor at the short path length end of their scale.

g. Temperature Controlled Cells

The effect of temperature on the measured value of absorbancy of a solution is in general small within the range of the usual laboratory conditions. For applications such as kinetic studies of enzymatic or other chemical reactions, control of temperature is required. Of especial interest to biologists is the observation that absorbancy of some high polymers, notably desoxypentosenucleic acid, is unusually sensitive to temperature. Cells have been made with surrounding jackets through which fluid can be circulated from a constant temperature reservoir. Soldered to the lamp house of the newer Beckman instruments are metal tubes through which water is circulated to dissipate the heat generated by the light source, and to prevent a rise in temperature of the sample compartment during prolonged observations. Beckman also makes a metal plate through which constant temperature liquid may be circulated; the plate is fitted onto the monochromator, and lies between the monochromator and the sample compartment. Somewhat more elaborate equipment for control of temperature in the sample compartment of the Beckman instrument has been described by Gibson (1949).

Equipment has been described in the literature (Passerini and Ross, 1953) to adapt the Cary for studies of absorption at reduced temperatures. The author has constructed an all-quartz Dewar flask that is equipped with optical windows for the sample and control beam of the Cary spectrophotometer. This device, filled with isopentane, chilled by liquid nitrogen, has been used to study absorption spectra over a wide range of temperatures (+20° to −198°C).

h. Flow Cells

Special cells are available, and others have been described, for the constant monitoring of the absorbancy at a single wave length of a solution flowing in a process stream or from a chromatographic column (Deutsch *et al.*, 1952). Flow cells have also been used for the study of the kinetics of some fast reactions (Chance, 1953).

i. Cleaning of Cells

It has been the practice in this laboratory to keep the silica absorption cells for ultraviolet spectrophotometry in a cleaning solution composed of one part nitric acid and three parts concentrated sulfuric acid. The cells are carefully rinsed with tap water, distilled water, 95 % ethyl alcohol, and finally ether. Drying of the cells is hastened by use of a filtered air stream. Care is taken of course not to handle the cells in such a way as to touch the optical faces.

In general the acid cleaning has given excellent results in our hands and has prevented an accumulation of substances on the surfaces of the cells that gradually changed the blank calibration. Difficulty has been encountered occasionally due to an accumulation of bubbles on the faces of the cells during a determination. This bubble formation was found to be due to a persistent thin hydrophobic film that resists removal by acid. We have found that Shamva[13] will completely remove the last traces of hydrophobic coating from the cells. After such treatment even solutions containing hydrogen peroxide give no trouble with bubble formation over prolonged periods of observation.

7. AUXILIARY EQUIPMENT

a. Paper Strip Attachment

There have been efforts to quantitate the amount of solute possessing ultraviolet absorption on a paper chromatogram or an ionophoretogram by measuring the absorbance of the paper strip containing the solute as compared with the absorbance of a control piece of paper. In this way a graph of ultraviolet absorption of the strip can be obtained as a function of the distance from the starting point of the solute. The area under such an absorption wave and the height of the maximum of the wave have been related to the total amount of solute present. Such determination then can be made quantitative.

Equipment for this procedure has been described for the Beckman DU (Paladini and Leloir, 1952; Brown and Marsh, 1953), for the Cary (Parke and Davis, 1952), and for the microspectrophotometers. The latter instrument is employed when narrow strips of paper are used to analyze extremely small amounts of solute (less than 1 μg).

Edstrom (1953) has described a photographic spectrophotometric method for the determination of the amount of ribosenucleic acid (RNA) extracted from a single neuron. This is accomplished by applying the droplet containing the extracted RNA to a Cellophane strip and allowing it to dry. The strip is then photographed with an ultraviolet microscope onto a calibrated photographic plate. Microdensitometry of the photomicrographic plate will yield an absorption wave similar to that obtained by the paper strip chromatographic method. When this wave is integrated in terms of absorbance, the total amount of RNA can be calculated. There appears to be no reason why this same procedure could not be carried out by photoelectric methods.

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[13] Obtainable from Golwynne Magnesite and Magnesia Corp., 420 Lexington Ave., New York.

b. Column Chromatography Monitor

The Beckman and the Cary have been adapted to serve as a continuous monitor of the ultraviolet absorbance of the effluent from chromatographic columns (Deutsch *et al.*, 1952). The spectrophotometers have been made to actuate the fraction collection system when a wave of solute has been collected, to change the solvent after a predetermined number of such waves have passed, to determine complete spectra at points selected on the basis of absorbancy of the eluate, and/or to periodically determine the absorbancy at a number of preselected wave lengths. Such an instrument would obviously save a tremendous amount in labor if a large number of

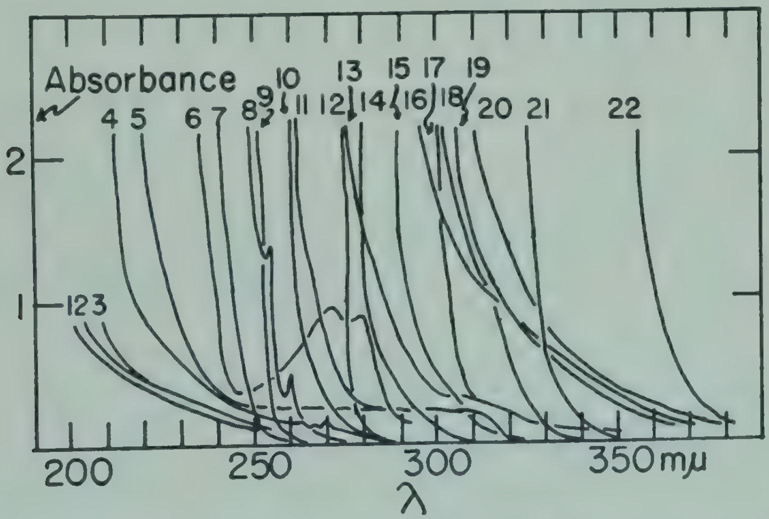

FIG. 35. Absorption spectra of a number of common solvents before purification. Pathlength in all cases, 1 cm. Reference solvent: glass redistilled water, 1 cm. 1–methyl alcohol. 2–ethyl alcohol. 3–ethyl ether. 4–isopropyl alcohol. 5–glycerine. 6–mineral oil. 7–chloroform. 8–ethyl acetate. 9–glacial acetic acid and formic acid. 10–carbon tetrachloride. 11–*n*-butyl alcohol. 12–benzene. 13–amyl acetate. 14–toluene. 15–xylene. 16–ligroin. 17–pyridine. 18–isobutyl alcohol. 19–ethyl oxalate. 20–amyl alcohol. 21–acetone. 22–benzyl alcohol.

chromatographic analyses were to be performed under essentially identical conditions of elution.

V. Solvents

The absorption spectra of a number of common organic solvents as taken directly from the reagent bottles are demonstrated in Fig. 35. It is to be noted that the 95% ethyl alcohol does not show a trace of benzene found in U.S.P. 100% ethanol. As mentioned earlier, these solvents can be used as cut-off filters. However, one should examine the absorption spectrum of each batch of crude solvent for there are tremendous variations from batch to batch.

The purification of solvents for spectroscopy is frequently quite a different thing from the purification of solvents as chemical compounds. Solvents as received from stock are sufficiently rich in a nominal compound to require no further extensive fractionation for most spectroscopic purposes. The main purpose of spectroscopic purification is to remove unwanted classes of compounds from the crude solvent. For example, the purification procedure for alcohol is designed to remove aldehydes and acids and similar compounds from alcohol, and the procedure for the purification of saturated hydrocarbons is designed to remove the corresponding unsaturated hydrocarbons.

Standard texts on spectroscopy contain references to methods of purification of certain common solvents. For convenience these references are repeated below in Table II.

An excellent method for purifying saturated hydrocarbons has been described by Mair and White (1935) and in more detail by Potts (1952). This method depends upon a strong affinity of silica gel for unsaturated compounds and hydroxyl compounds. The procedure is simply to prepare a column 1 inch in diameter, approximately 4 feet long, and packed with silica gel[14] through which the crude hydrocarbon is allowed to percolate without use of additional pressure. The absorbance at 2200 A is measured against water and the solvent is collected in 100-ml fractions. All fractions having less than a preselected maximum absorbancy are pooled as are those which have an absorbancy greater than this value. Repeated runs are necessary to reduce the absorbance of the second pool to that of the first. The number of runs varies from compound to compound and also from batch to batch in the same compound. This method has served extremely well and is a rapid, efficient, almost automatic method of purifying saturated hydrocarbons without previous treatment with sulfuric acid or fractional distillation. The efficiency of a given column depends upon the relative content of compounds of varying degrees of saturation, since com-

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[14] Available from the Davidson Chemical Corporation, Baltimore, Md. Commercial Grade, Mesh Size 20-65.

TABLE II
Purification of Solvents

Solvent	Reference
Methanol	Morton and Mark (1934)
Ethanol	Castille and Henri (1924)
Butanol Propanol	5gm Zn dust, 10gm KOH, 10 ml H_2O, 1 liter stock butanol or propanol. Reflux 6 hrs. Distill off alcohol under reduced pressure.
Ethyl ether	Castille and Henri (1924)
Dioxane	Hess and Frahm (1938)
Chloroform	Silica gel treatment (see text)
Carbon tetrachloride	Twyman and Allsopp (1934)
All saturated hydrocarbons	Mair and White (1935); Potts (1952)

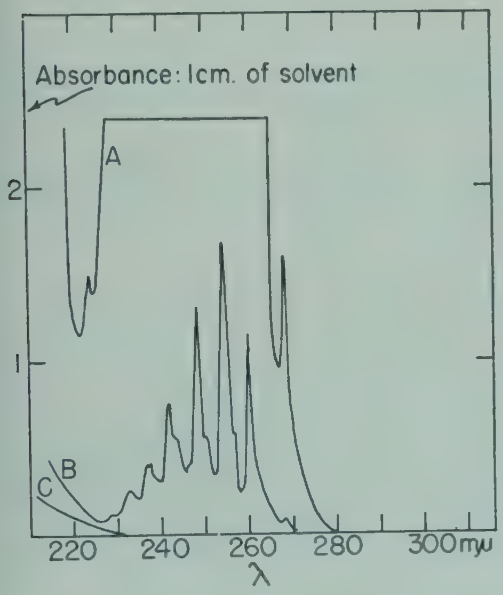

FIG. 36. Purification of hexane by passage over silica gel: A. Crude hexane. B. After one passage through silica gel column. C. After second passage through a silica gel column.

pounds of low degree of unsaturation will easily elute from the silica gel and hence quickly appear in the effluent. Figure 36 shows the efficiency of this method of spectroscopic purification of hydrocarbons. In our hands it has produced a solvent of significantly better transmission than that produced by conventional sulfuric acid treatment followed by careful fractional distillation in a 17-plate Brun fractionating column.

VI. Buffers for Ultraviolet Absorption Spectrophotometry

In general buffers which do not employ conjugated compounds such as Veronal and phthalate are satisfactory for ultraviolet absorption spectrophotometry in relatively low concentrations, i. e., 0.1 *M* or less. Care must be exercised, however, in the use of highly alkaline buffers since the absorbancy is frequently much greater than one would predict on the basis of the chemical composition of the buffer. Furthermore, absorption cells of silica are easily attacked by strong alkaline solutions. The following buffers are in general satisfactory: NaCl-HCl, formic acid, citric acid, malic acid, phosphate, tris, borate, and glycine buffers.

VII. Calibration of the Ultraviolet Spectrophotometer

As with any measuring instrument two factors must be determined for each individual spectrophotometer: Precision (reproducibility) and accuracy (proximity to the truth). These factors must be determined for both measured variables, wave length and absorbance (transmittance). The precision and accuracy must be determined both initially and periodically during the life of the instrument.

1. WAVE LENGTH CALIBRATION

The nominal wave length of the geometrical center of the band of radiation emerging from the exit slit of the monochromator is usually read from a dial or a scale inscribed simply with numbers bearing no relation to the wave length, or inscribed with numbers very close to the actual wave length. In either case the numbers read from the instrument must be related to the true wave length.

The usual technique of wave length calibration in the ultraviolet involves the use of a mercury vapor lamp as the source of radiation for the spectrophotometer replacing the tungsten or hydrogen source furnished. The instrument is adjusted to yield maximum resolution according to directions usually furnished by the manufacturer. Then, using the instrument as a detector of radiant energy, the known emission lines of mercury are sought and the dial or scale reading for maximum energy is recorded for each of the known mercury lines. A plot is then made of λ_{known} *vs.* λ_{red} and from this a correction table is made.

This is a relatively simple procedure but it has a few drawbacks. With instruments of high resolution such as the Cary, more emission lines can be found than can be identified with certainty from the available tables. This problem has been dealt with by Heidt and Bosley (1953), and their paper contains a valuable listing of the detectable emission lines from an easily available mercury discharge source.

Recently another method of wave-length calibration has been revived and used by Heidt and Bosley (1953). This method is based on the interference in a monochromatic beam of light which traverses two closely spaced, partially reflecting surfaces held parallel to one another and separated by a known small distance. Directions are given in this paper for

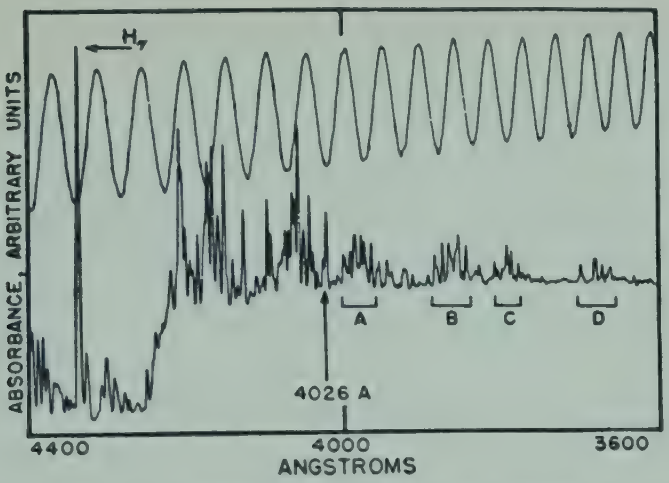

FIG. 37. Absorbance wave of interference pack as recorded by a Cary spectrophotometer. Also shown is a portion of the spectrum of the hydrogen lamp which serves as the source of continuous light for the ultraviolet range. The lines from this source may be used to determine the wavelengths of the maxima and minima of the absorbance wave. Reproduced from Heidt and Bosley (1953).

the construction and calibration of such a device. The result of a calibration run for the Cary is shown in Fig. 37.

The precision of the wave length scale is determined by repeated calibration runs under identical conditions of resolution. At this time one should determine the effect on the calibration of traversing the spectrum in opposite directions. The difference in the calibration runs in opposite directions will give a record of the amount of "backlash" in the mechanical system for wave length drive, and will furnish a basis for an estimation of wear in the system as the instrument ages. In practice, of course, spectra should always be determined in one direction.

Most biological work does not require the very high degree of accuracy and precision required for emission spectroscopy, and for the refined spectrophotometric studies of chemical physics (±1.0 A or frequently much less) although this is obtainable with some instruments discussed here. It is unusual for the biologist to encounter compounds possessing many sharp bands in which small changes of wave length are significant (see, however, the discussion on protein absorption).

In general, ultraviolet spectrophotometers are reasonably accurate and precise in the regions between 2400 and 3000 A where the dispersion of quartz is good, and the difference in the refractive index between different batches of quartz is very small. Large wave-length correction factors will frequently occur towards the extremes of the wave-length range because of low dispersion as the visible spectrum is approached and because of variation in refractive index from prism to prism at the short wave-length end of the spectrum.

2. Absorbance (Transmittance) Calibration

The problem of the absolute accuracy of absorbance measurements has been of constant interest to the users of photoelectric and photographic spectrophotometers. The principle of calibration of the absorbance scale is simple: a variable known fraction of the incident light for any given narrow band of radiation is removed, and the response of the instrument noted. The discrepancy between the actual response of the instrument and the response predicted from known fractional light loss is then the error in absorbance. In practice this is not always accomplished in an absolute fashion. Before discussing the methods of practical calibration of the absorbance scale it is appropriate to discuss some of the more important factors which influence absorbance measurements.

a. Stray Radiation

Stray radiation is defined as light emerging from monochromator of wave lengths other than those included in the band of wave lengths determined upon geometrical grounds by the setting of the wave length dial and by the setting of the slit width.

The practical effect of stray radiation is pictured in Fig. 38. It can be seen to reduce the absolute absorbancy and/or to produce false absorption maxima. This effect is most commonly seen in the ultraviolet near the low wave length limit of the spectrophotometer. This effect can be expressed

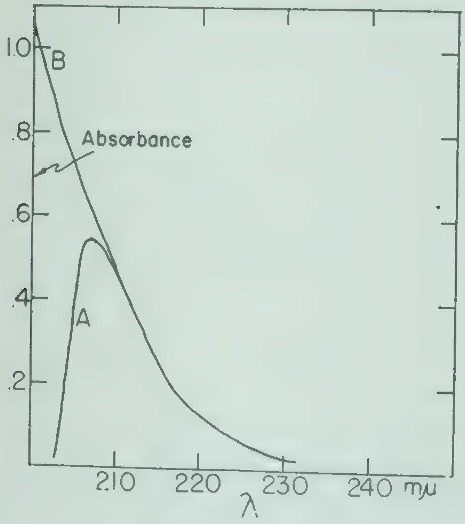

FIG. 38. Absorption spectrum of 0.013 M glycine: A. Showing false maximum due to scattered light. B. Showing the result after cleaning the instrument and replacing the mirrors. Redrawn from Saidel *et al.* (1951).

mathematically as follows:

$$10^{A'} = \frac{x + 1}{10^{-A} + x} \tag{1}$$

where A is the true absorbancy and A' is the found absorbancy and x is the fraction of the effective stray radiation incident on the sample. The reason for the rapidly increasing effect of stray radiation in producing errors in absorbancy as one approaches the short-wave-length limit of the monochromator can be seen in the fact that the proportion of stray radiation to nominal radiation (x) will increase rapidly due to the rapid fall in the intensity of nominal radiation.

A number of methods of testing for stray radiation have been proposed. The indirect test described by Beaven and Holiday (1950) is relatively simple and easy to carry out. It is somewhat more stringent than may be required for practical spectrophotometry but one can be assured that the performance of the spectrophotometer will be as good as or better than the test would indicate. This method of assessing stray radiation was also used in principle by Saidel *et al.* (1951a) in the investigation of peptide absorption described earlier.

The technique requires simply a cut-off filter which has an absorption curve rising from an absorbance of approximately zero at 2300 A to an absorbance of five or greater at 2200 A. The apparent absorbance (A') is measured from 2200 to the short wave length limit of the instrument. The per cent scattered light of wave lengths greater than 2300 A can then be calculated either from the relation given by Saidel or as given by Beaven and Holiday. It follows from Eq. 1 above that as A approaches infinity then for practical purposes $10^{A'} = \frac{x + 1}{x}$. When x is determined as a function of wave length a graph of A vs. A' can be constructed as a family of curves for each wave length or a set of tables may be calculated and the appropriate correction made from these tables. Values of x must be redetermined periodically for reasons enumerated above. By an appropriate choice of filters (see page 164) one can determine the fraction of stray radiation for the whole range of the spectrophotometer.

The most common sources of stray radiation in monochromators and spectrophotometers are reflections from the entrance of the Littrow prism (Hammond and Price, 1953), reflection from baffles, dust on optical surfaces, and poor quality of reflecting surfaces.

The problems of stray radiation in spectrophotometry have been discussed at length in the following references: Hogness *et al.* (1937); Storey *et al.* (1950); and Opler (1950).

b. Non-linearity in the Electrical Components

The electrical circuits of modern commercial spectrophotometers are linear to a high precision. There has been reported a case of non-linearity of the photocells of the Beckman which manifests itself as a change in extinction coefficient of a standard when an old photocell was replaced with a new cell (Cannon and Butterworth, 1953). This is not a common phenomenon with the ultraviolet sensitive phototube, but occurs to some degree with all red sensitive tubes used by Beckman (Goldring *et al.*, 1953). The over-all linearity of the electrical system can be checked by use of screens as described by Heidt and Bosley (1953). In this test two appropriately selected screens are measured first singly and then in tandem at appropriate orientation for a series of slit widths. This, in effect, measures absorbance of a neutral standard as a function of the intensity of the incident light.

c. Effect of Slit Width

The statement of Beers' law (Chapter 3) is made for the condition of monochromatic light which can only be approached by a line source. In a practical spectrophotometer one generally uses a band of radiation which may be from a few angstroms to hundreds of angstroms wide. Commercial spectrophotometers are usually accompanied by a curve or a table which gives the band width (absolute or half maximum intensity band width) for a given opening of entrance and exit slits. It can be seen intuitively that the measured absorbancy will be an integrated value of absorbancy over the whole band of radiation. Unless the change of absorbancy with wave length ($dA/d\lambda$) is constant there will be an effect on the measured absorbancy when the slit width is varied. It should also be pointed out that in the region of the spectrum in which the over-all response of the instrument may be changing rapidly with wave length the effective band center in terms of instrument response may be displaced considerably from a geometrical band center thus leading to errors in reporting absorbancy in absolute terms. This effect would differ from phototube to phototube and also from instrument to instrument, and would be most noticeable at wide band widths.

The effect of varying slit width for ultraviolet absorption work has been reported by a number of people (Eberhardt, 1950; Brodersen, 1954). For example, Hogness *et al.* (1937) demonstrate that the molecular extinction coefficient for the 2540 A band of benzene in isoctane will drop from 212 ± 2 to 136 ± 2 as the band width is increased from 2.8 A to 20.0 A. Recording spectrophotometers which employ a servomechanism to maintain constant energy in the reference circuit by increasing the slit width as the energy incident upon the phototube decreases have peculiar problems of their own. If one imagines that the solvent in the reference beam contains a compound such as benzene, it will be seen that the slit width will increase and decrease as the instrument scans over the absorption bands of benzene. This of course produces a fluctuation in absorbance accuracy and must be considered in the use of such instruments. Forstner and Rogers (1953) have reported that the Cary spectrophotometer can actuate the slit mechanism with sufficient rapidity to keep up with the absorption spectrum of benzene in the control beam up to a scan ratio of 5 A/sec even when the absorbancy at the highest benzene peak is 1.5.

d. Optimum Concentration

The existence of an optimum concentration is the natural consequence of instrumental uncertainties and of the absorption law. The problem of optimum concentration has been considered by a number of workers and also elsewhere in this volume (Chapter 3). It is not peculiar to the ultraviolet portion of the spectrum and will not be considered further here.

e. Instability of Electrical Components

In any single beam instrument the accuracy of a determination depends, among other things, upon the stability of the whole electric circuit, including the light source, the phototube, the load resistor, the amplifier, the indicating device, and the power supplies for all these components. The most common source of major instabilities is found in loose connections in the high current-low voltage leads, high humidity affecting the high impedance amplifier input circuit, and instability in the phototube and amplifier power supply. These instabilities may be manifested as either a relatively slow drift or a quick, random fluctuation of the zero indicator. The slow drift is probably due to changes in the power supply such as deterioration of the batteries. High humidity which affects the high impedance input of the amplifier, will also produce a slow drift in the balance point. Quick, random movements of the above indicator usually denote a loose connection or poor switch contacts.

In the Beckman DU the effect of old C batteries can increase the per cent relative error by a factor of 5. Other electrical instabilities will have similar effects. In addition to the increase in instrumental error, there is also an increase in the subjective component of error due primarily to the annoyance of operating an unstable instrument and to the large increase in time required to complete a series of readings. The importance of the reduction of the subjective source of error in absorbance measurements by careful maintenance of the instrument cannot be overemphasized.

f. Cell Differences

The difference in absorbance between a group of cells which is due to differences in the homogeneity and quality of the quartz is usually not significant at wave lengths greater than 2500 A provided the absorbance of the sample is in the optimum range. Below 2500 A and for lower absorbance readings the cells must be carefully calibrated by wave length. This is done by reading the solvent filled cells against one another throughout the wave-length range of interest. It is difficult to give any precise figures for this factor since conditions must be specified and may change with time.

g. Instrument-to-Instrument Variation

The calibration of the absorbance scale often depends upon the comparison of the absorbance of a given standard sample as obtained by the instrument to be calibrated with the average value obtained for the same sample on a large number of other instruments. It is necessary to have some idea of the coefficient of variation from instrument to instrument in order to assess the significance of the difference in absorbance found between the standard value and the value given by the instrument under consideration. Gridgeman (1952) discusses this form of error.

This point has been reported on by Caster (1951) for the Beckman instrument. He calculated the results of two other workers and arrived at values of 1 % and 5 % coefficient of variations. Brode *et al.* (1953) give the results for a similar test on thirteen Beckman instruments and obtained values ranging from 2 % to 50 % depending on the value of the absorbancy. An over-all instrumental error of 1.6 % has been reported by Gridgeman (1951) for 75 spectrophotometers of Beckman and Unicam variety. Goldring *et al.* (1953) report a value of 0.23 % coefficient of variation for a group of 18 Beckman spectrophotometers containing both new and used instruments.

For the Cary spectrophotometer, Brode *et al.* (1953) report, on the basis of 13 instruments, a coefficient of variations of from 1.4 % to 30 % for an absorbancy range of .993 to 0.0556 respectively. The precision of a single instrument is better than this (Forstner and Rogers, 1953). Brody emphasizes that for the ratios of absorbance the instrument-to-instrument percision is ten times better than that for absolute measurement. Hence ratios can be readily compared between the instruments or when the log absorbance slide wire is employed (such a slide wire is available for the Cary spectrophotometer), the spectra can be overlaid for comparison with excellent precision. (See also Vandenbelt (1954).)

h. Practical Methods of Absorbance Calibration

(1) Standard solutions. The most carefully worked out solution standards are those of potassium chromate. When carefully prepared solutions

of recrystallized potassium chromate in alkaline solution are prepared according to the directions given by Haupt (1952) the differences between the absorbancy obtained with the instrument being calibrated and the standard values given can be taken as indicative of the over-all accuracy of the absorbancy scale. Because this standard solution is not a neutral absorber, due regard must be given to the fact that the accuracy is influenced by the factors given above, and further that these factors vary with the absolute absorbancy level and also may vary with time.

Absorbancy calibration in the ultraviolet by use of standard solutions is a practical over-all method of calibration and the reader is urged to consult the reference given above.

(*2*) *Standard screens.* A method for removing a known fraction of a beam by an opaque substance is afforded by wire screens. A recent careful study of the application of wire screens as absorbancy standards has been made by Heidt and Bosley (1953). They found that the screens were not completely neutral but that the change in absorbancy was not great and could be represented by a linear equation. The use of these screens is recommended for the ultraviolet. The above authors report that the probable deviation of one Cary recording spectrophotometer as 0.74 % from the value calculated by the percentage of the area of the beam blocked out by the wires of the screen. The deviations were found to be independent of wave length and of absorbancy up to a value of 2.8. These screens may also be used to calibrate the linearity of the photocells as noted on page 192.

It should be pointed out that the indicated accuracy of the absorbance obtained with neutral density devices should not be taken as a measure of accuracy of absorbance measurements of substances with a steep slope of absorbancy *vs.* wave length. Neutral density filters may also give a false low value for the per cent scattered light.

When using wire screens or other neutral density absorbance standards for the calibration of automatic recording spectrophotometers it should be remembered that the neutral density characteristics of these standards will give no indication of the ability of an automatic recording device to follow rapid changes in absorbance.

When properly used neutral density devices such as the screens described by Heidt have a definite advantage. The absorbance of the screens can be calculated, and hence merit careful consideration for determination of absorbance accuracy with a greater degree of independence from the multiplicity of factors affecting the over-all error in absorbance determination.

VIII. Literature

1. GENERAL

No effort will be made to survey completely the literature for reference to ultraviolet absorption data. Rather, texts, compilations, reviews, and periodicals which will serve as a guide to the worker in search of spectral absorption data will be noted.

a. Texts

(1) Spectroscopy. Standard texts which have been recently published or revised include Brode (1943), Gibb (1942), Harrison, *et al.* (1948), and West (1949). These books contain many references to papers dealing with developments in the component parts of the spectrophotometer which will be of use to one contemplating the construction of a spectrophotometer.

(2) Molecular structure and spectra. For a general discussion of the electronic nature of the chemical bond, molecular structure, energy levels, and spectra the following works are suggested: Coulson (1952), Pauling (1945), Hinshelwood (1951), and Branch and Calvin (1941).

b. Reviews

There are a number of excellent reviews on the application of absorption spectroscopy to problems of chemistry. A few of these are given for the interested reader: Lewis and Calvin (1939), Sponer and Teller (1941), Bowen (1943), Braude (1945), and Ferguson (1948).

The Annual Review of Physical Chemistry has regularly contained a chapter on spectroscopy since publication began in 1950. In these chapters the emphasis is on the relation of molecular structure to light absorption.

c. Periodicals

(1) The journal Spectrochemica Acta has recently been attempting to include more papers on absorption spectroscopy in its issues than had been included in earlier years when it was primarily concerned with emission spectroscopy. The most recent issues still contain a preponderance of papers on the latter subject.

(2) Applied Spectroscopy is published irregularly by the Society for Applied Spectroscopy. The majority of papers are concerned with emission spectroscopy.

(3) The British Bulletin of Spectroscopy sponsored by the Industrial Spectroscopy Group of the Institute of Physics, The Photoelectric Spectrophotometry Group, The IR Discussion Group, and The Physical Methods Group of the Society of Analytical Chemists is published quarterly. This publication contains a valuable survey of papers on all forms of spectroscopy which have appeared in the general literature in the quarterly interval. The survey does not appear to include the literature which is principally biological.

(4) The Photoelectric Spectrophotometry Group Bulletin has contained many fundamental papers on instrumentation. It is obtainable through

The Honorable Secretary: W. H. Storey, Arbury Works, Arbury Road, Cambridge, England.

(5) *An American pamphlet, Spectroscopia Molecular*, is published monthly in "Interlingua" by Spectroscopy Laboratory, Illinois Institute of Technology, Chicago 16, Illinois. It is primarily a news letter, but original papers and surveys of literature are regularly included.

(6) *The following periodicals* publish many papers which contain spectral data of value to the biologist:

J. Am. Chem. Soc.
J. Chem. Soc.
Ann. Chem.
Analyst
Chem. Revs.
J. Chem. Phys.

The journals of biological chemistry are publishing a steadily increasing number of papers in which absorption spectroscopy plays a major role.

(7) *The periodicals publishing papers* concerned primarily with instrumentation in spectroscopy include:

J. Opt. Soc. Amer.
Rev. Sci. Instr.
J. Sci. Instr.

(8) *In Chemical Abstracts* the papers in which the major emphasis is on spectroscopy are abstracted under the head, "Electronic Phenomena and Spectra."

2. Compilations of Spectral Data

a. *Standard Compilations*

Included in this category are collections of data which are augmented periodically with additional information.

(1) *International Critical Tables:* Carr *et al.* (1929); Holmes (1931).
(2) *Tables Annuelles de Constantes et Donnees Numerique:* Henri (1932) and Magat and Maier (1943).
(3) *Landolt-Bornstein, Physikalische-chemische Tabellen* (1951)
(4) *Tabulae Biologicae:* Ellinger (1937, 1938)
(5) *Beilstein's Handbuch der Organische Chemie*, 4th ed. Contains many literature references to spectral data.
(6) *American Petroleum Institute:* Issues ultraviolet, visible, and infrared absorption data as well as Raman data on compounds of interest to the petroleum industry of which many are also of biological interest.

b. *Miscellaneous Compilations*

Under this heading are included compilations of data which have appeared in the literature and which cover many classes of compounds.

(1) Loofbourow (1943) discusses the application of physical methods, including spectroscopy, to the chemistry of vitamins and hormones. The same classes of compounds are also covered by Brode (1944) and by Morton (1942).

(2) Loofbourow (1940) and Anslow (1945) give a number of spectra, tables of data, and literature references to spectra of compounds of biological interest.

(3) Miller (1934) has collected a large number of spectra of natural products in this valuable volume.

(4) Jones (1943) and Friedel and Orchin (1951) have collected the ultraviolet spectra of a large number of polycyclic aromatic hydrocarbons.

(5) Aronoff (1950) has compiled data on compounds related to chlorophyll.

(6) *Analytical Chemistry* publishes at intervals compilations of literature references on ultraviolet absorption spectroscopy (other regions of the spectrum are also covered in separate papers). The literature references are segregated according to the compounds examined. The most recent of these reviews are contained in Vol. 24, No. 1, 1952, and Vol. 26, No. 1, 1954.

c. *Specific Compilations*

Here are included compilations of data on single classes of compounds of biological interest.

(*1*) *Steroids.* Some recent reviews on steroids which give a large number of spectra of the sulfuric acid chromogens, the unaltered molecule, or both, are Dannenberg (1940), Bernstein and Lenhard (1953), Dorfman (1953), Zaffaroni (1953), Axelrod (1954), and Bates (1954).

(*2*) *Proteins.* Beaven and Holiday (1952) and Dannenberg (1951) have recently reviewed and discussed the spectroscopy of amino acids, peptides, and proteins. These papers contain a large number of references to the recent literature.

(*3*) *Nucleic acids and related compounds.* Unfortunately the literature concerning these important compounds has not been reviewed with the purpose of pulling together and evaluating the many papers on the ultraviolet absorption spectra of these compounds. No attempt will be made to do so here, but some references will be given to help the reader into the literature on this subject, which has some puzzling aspects (see page 143).

(a) Purines and Pyrimidines: Loofbourow (1940), Cavalieri *et al.* (1948), Cavalieri and Bendich (1950), Shugar and Fox (1952), Wyatt and Cohen (1952), and Sinsheimer (1954).

(b) Nucleosides: Manson and Lampen (1951), Cavalieri (1952), Fox and Shugar (1952), and Fox *et al.* (1953).

(c) Nucleotides: Volkin and Carter (1951), Ploeser and Loring (1949), Fox *et al.* (1953), Sinsheimer (1945).

(d) Oligonucleotides: Sinsheimer (1954)

(e) Polynucleotides: Kunitz (1950), Frick (1951), Tsuboi (1950), Laland *et al.* (1954), Thomas (1954a,b).

(*4*) *Fatty acids and lipids.* Burr and Miller (1941).

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Chapter 5

INFRARED SPECTROPHOTOMETRY

Carl Clark

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I. Introduction

1. Topics Not Considered

This chapter concerns those aspects of infrared techniques which I as a biologist have found or expect to find useful. It is primarily directed to those who have not had experience with these techniques.

Infrared radiations are electromagnetic radiations of wave lengths longer than visible red light (about 0.75 microns) and shorter than the electronically presently produced "microwaves" (about 1000 microns). Half of the sun's emission which reaches the earth is infrared radiation (between 0.75 and about 3 microns wave length). Bodies which are less hot such as hot stoves or warm blooded animals emit a greater proportion, to all, of their radiant energy as infrared radiations, or "radiant heat." An animal is continually adjusting to its radiation environment; in the case of a nude human in an environment at 23°, infrared radiation may account for 60% of the basal heat loss (DuBois, 1938). A human being can detect a skin surface temperature rise of 0.0008°C per second within 3 seconds (Hardy and Oppel, 1936). Further aspects of heat physiology are not discussed in this chapter. A relation has been suggested but not yet adequately demonstrated between infrared emission and absorption and the smell sensation (Dyson, 1937; Beck and Miles, 1947; Forrester and Parkins, 1951). Pretreatment with filtered one micron near infrared radiation has been found to increase the number of chromosome rearrangements produced by subsequent x-radiation (Kaufmann *et al.*, 1946). These topics as well as near infrared photography and radiation thermometry are not discussed. Raman spectra, although involving similar molecular vibrations to those responsible for infrared spectra, are obtained by quite different techniques in which the scattered radiation, of different wave length from the source, is observed, generally in the visible radiation region. These techniques are not discussed; see for example Harrison *et al.*, (1948), or Hibben (1950).

Hot bodies may emit energy at the various wave lengths, which is proportional to that given by the Planckian or black body distribution curve, the proportionality constant being called the emissivity of the body. If the emissivity is indeed a constant at all wave lengths one may speak of the emission as non-selective. If the emissivity, or also the absorption, is not constant at all wave lengths, one may speak of selective emission, or selective absorption. It is this selective aspect of, particularly, infrared absorption that will be emphasized in this chapter. This field of study, which requires the use of resolved infrared radiations that are quantitatively measured, is called infrared spectrophotometry.

2. The Advantages and Limitations of Infrared Techniques

As discussed below, the magnitudes of absorption and the wave lengths or frequencies of the selective infrared absorption bands characterize the vibrational and rotational energy states of the molecular structure of the sample. If the spectra are sufficiently complex, with many sharp bands, they constitute "fingerprints" of the samples; spectra with broader bands may not as accurately characterize the molecular structure involved. It is this aspect of infrared spectra, as characteristic physical properties of particular molecular structures, on which are based the following applications and advantages.

Physical methods of chemical analysis have a general applicability to unknown samples, allowing systematic and inclusive analyses in a more direct manner than chemical methods, which involve specific reactions for specific groups and therefore require some prediction as to unknown properties in order to decide which tests to apply. Infrared absorption and x-ray diffraction techniques share advantages over other physical methods in that they provide tens or even hundreds of numbers characteristic of particular molecular types, allowing specific identification; many samples may have the same boiling point. These methods are readily mechanized and may be utilized without detailed instrumental training, thanks to the commercial instrument developments of the last few years. Detailed infrared spectra may be obtained with samples in gas, liquid, or solid form; detailed x-ray diffraction interpretations require crystallinity. Polarized infrared spectra, as well as x-ray diffraction techniques, may indicate molecular orientations (see Section III).

Infrared spectra may be obtained with a few milligrams of sample, or with care with a few micrograms. This method of measurement alters the sample being measured very slightly and temporarily; samples may be recovered unchanged. Infrared photochemical reactions seem rare, and increased thermochemical reactions or changes of state are usually negligible or may be minimized by putting the sample after the monochromator. Infrared spectra may be obtained in less than an hour to a few hundredths of a second, depending on the detail and method of recording. Three or four spectra an hour, from 2 to 16 microns (5000 to 625 cm^{-1}), may be obtained with present commercial instruments.

The specificity of infrared spectra of small molecules is impressive. It appears that most organic molecules of molecular weight below about 300, and many inorganic ones, have characteristic infrared spectra which allow their specific identification from catalogs of the spectra of known substances. Steriosomers, including *cis-trans* isomers, a DL- from an L-amino acid (Darmon *et al.*, 1948), and alpha- from beta-sugars (Whistler and House, 1953), different inorganic salts of the same organic acid or base, and even different hydrates, and polymorphs (Kendall, 1953) of an organic molecule otherwise the same may be distinguished. Decisions as to the predominate tautomeric forms of a molecule, hydrogen bonding, and other inter-molecular aggregations and changes of state may be assayed. The replacement of hydrogen by deuterium in a molecule produces characteristic infrared spectral changes (Halverson, 1947), which can be used to identify those bands involving hydrogen vibrations. The mass changes in other isotope groups is proportionally much less, but the spectral differences have been detected in some cases.

It is this very specificity of infrared absorptions which creates a major difficulty. Solvents absorb, and so must be considered carefully in making an analysis. Water absorbs strongly, limiting studies of aqueous solutions. All or most components of a mixture absorb, with a great overlapping of bands. One cannot readily select one component which alone will affect measurements, although with gas analyzers this may be done with some success (see below). Infrared spectra of biological tissues give little indication of the great chemical complexity because of the overlapping of the many absorption bands. If the separate components cannot easily be chemically distinquished, infrared spectra may still be the best method of analysis. But chemical methods have their great advantage if the desired component can be identified by a reaction which is unaffected by surrounding materials. Thus if one is interested just in the urea content of blood, a chemical method is much simpler than infrared methods. But as we become interested in complete molecular descriptions of blood, or other tissues, secretions, extracts, or hydrolysates, or of any other complex chemical mixture, systematic infrared analysis following systematic fractionation, as by solubilities, step-wise hydrolysis, and chromatography, offers advantages over the great multiplicity of chemical reactions which would have to be applied to obtain the same results. For example, one can visualize a single chromatographic and infrared procedure to provide complete small molecule blood chemistry analyses now given by many separate chemical analyses.

Similar chemical groups in different parts of a large molecule may have slightly different "molecular environments" (Weiss, 1949), so that the vibrational energies and hence wave lengths of absorption bands attributable to them may be slightly different. Macromolecules in general have broad absorption bands, which may be interpreted in terms of the elementary group analysis method (see Section III) as arising from an overlapping of these many absorption bands. Hence as molecular weights increase the infrared spectra at first show a roughly corresponding increase in complexity but then become less complex again, with broad absorption bands. Large molecules have more unknowns (possible structural complexities) than the equations relating them (the few broad absorption bands); their infrared spectra cannot at a glance safely constitute unique identifications. It is worth emphasizing that chemists do not yet have much information as to the spatial structure and variability of macromolecules in similar positions or serving similar functions in related biological species. It may be that nature is not as prolific in turning out strings of amino acids, for example, as a mathematically inclined chemist, so that the infrared spectra may be sufficiently complex in many cases to distinguish the structures which actually occur in nature. In making such decisions one should utilize not only the positions of the absorption bands but also their shapes and absorptivities. "Large" in the sense of this paragraph seems to be about molecular weight 300; steroids (molecular weights 300–400) still give readily distinguished spectra, but oligopeptides above molecular weight 300 must be examined more carefully for differences, and nucleotides (molecular weights over 300) have fewer bands than nucleosides (molecular weights under 300).

Infrared spectra can be of great help in detecting and identifying impurities in a sample, for the impurities produce their effects on the spectra whether or not their presence is expected. However the impurities are not emphasized in the spectra, as they might be in certain specific chemical reactions, so that an impurity molecular type which is less than 1 % of the total sample, or even 5 % if the spectrum is poor, may be missed. Under ideal conditions, a contamination of 0.01 % has been detected by infrared spectra. Again, for critical studies one must observe not only the positions of the absorption bands but also their shapes, such as the appearance of slight plateaus. Quantitation of impurities has errors perhaps an order of magnitude worse than the limits of detection, with a minimum error being set by the noise level of recording of the spectrum.

Infrared spectra may be used to follow the dynamics of a chemical reaction, for many of the components may be specifically identified in the changing spectra. A notable example is the study of the chemical changes in an explosion (Bullock and Silverman, 1953), using oscilloscope recording of the infrared spectra. Biological reactions will be difficult, but not impossible

(Blout and Lenormant, 1953), to study because of the strong absorption of water.

Perhaps 10 % of infrared studies currently being published involve quantitative analyses. The accuracy can approach that of ultraviolet or visible spectroscopic techniques, with however the added difficulties of the greater "noise" in the usual infrared spectra and the great overlapping of the absorption bands of the components. The great advantage of the infrared method is that many components may be measured which have no characteristic ultraviolet or visible absorptions. Automatic process control utilizing continuous infrared spectra of fractionation products is becoming an important technique in the petroleum industry. Important biological engineering applications might be foreseen, as in controlling ideal conditions for the growth of yeast in the beer industry, or of molds in the preparation of antibiotics. Randall's group at Michigan, for example, have followed characteristic changes in the infrared spectra of extracts of bacterial cultures to provide information for controlling the bacterial growth (Randall and Smith, 1953).

From the infrared spectrum one can attempt an assignment as to the structure of the molecule even if the spectrum is not included in the catalog of knowns. Chemical groups usually have characteristic regions of absorption (Section III), so that many possibilities as to groups present in an unknown molecule may be eliminated by the study of its infrared spectrum. At the resent time one cannot usually estimate the complete structure from the spectrum; the possible structures are obtained or synthesized, then the spectra of known and unknown are directly compared. It may be hoped that before too long all molecules known to man will be included in an international catalog of infrared spectra, and as new molecules become known their spectra will be procedurally added to this catalog, thereby decreasing the anguish in determining the structure of a molecule which, quite probably for most of us, has been dealt with previously.

The physicists and physical chemists utilize infrared spectra for analyses of energy states of molecules from which they attempt to predict the details of molecular dynamics, including atomic density clouds, and interatomic distances, angles, and forces. It is their goal to perfect the theory and techniques of wave mechanics to be able to predict the energy states and behaviors of molecules of assigned structures, and to be able to assign such structures on the basis of data from standard experimental procedures. Thus far it has been possible to "explain" the spectra of a number of simpler molecules, involving solutions of the wave equations for these molecules utilizing a consistent set of assumptions and calculations based on these assumptions. Herzberg (1945) reviews interpretations of linear molecules (CO_2, C_2H_2), symmetric top molecules (C_2H_6, NH_3, CH_3Br), asymmetric top molecules (with symmetry less than a threefold rotation axis; HOH and most other molecules) and specific examples up to molecules with 12 atoms, although in many cases even these interpretations are partial. Frequently measurements of the rotational fine structure which is superimposed on the vibrational absorption bands are utilized. As molecular weights increase, moments of inertia of the molecule increase and correspondingly the rotational fine structure lines become closer together until they are no longer resolved even with the best grating spectrographs (Nielsen and Oetjen, 1950). Moreover, in the liquid or solid state, discrete rotational energy levels are in general not observed. Wave mechanics theory and techniques then are not yet adequate for a complete dynamic description of most biochemicals, although partial interpretations and structure suggestions may be presented.

It may be emphasized, with Rawlins and Taylor (1929), that infrared spectra present a dynamic approach to molecular structure and that the mean positions ("static") description is subsequently calculated. X-ray diffraction techniques directly measure these mean atomic positions, but it is interesting to note that molecular vibrations affect the x-ray data (Lonsdale, 1942), although theoretical interpretations are not yet sufficiently advanced to afford predictions of the infrared positions of the vibrational absorption bands from the x-ray data.

3. TERMINOLOGY

The terminology recommended by the Optical Society of America and reviewed in the chapter in this book by Dr. Hiskey will be followed. The basic relations

$$\nu = \frac{c}{\lambda}\,; \qquad \sigma = \frac{1}{\lambda}$$

should be readily utilized, in which ν is the frequency (sec.$^{-1}$ or waves per second), c is the velocity of light (2.998×10^{10} cm per second), λ is the wave length (usually given in microns in the infrared region, but which must be converted to centimeters for use in the above equations), and σ is the wave number (cm^{-1}, reciprocal centimeters, or waves per centimeter). The symbol σ is recommended by the Joint Commission for Spectroscopy (*J. Opt. Soc. Amer.* **43,** 411, 1953) to replace the older $\bar{\nu}$, which was sometimes written as ν and hence could be confused with frequency. As an example, 10 μ (microns, wave length) is equivalent to 1000 cm^{-1} (waves per centimeter, wave number). It should be noted that the wave length of a radiation varies inversely as the refractive index of the medium through which it passes. The wave lengths and wave numbers of the radiations referred to in this chapter are those that would be measured in a vacuum.

Much of the infrared work has been presented on a wave length scale, although there now appears to be a predominating use of the wave number scale, with its direct proportionality to quantum energy by the relation

$$E = h\nu = hc/\lambda = hc\sigma,$$

in which h is Planck's constant, 6.624×10^{-27} erg sec. I find Barlow's Tables (Comrie, 1947), with reciprocals of all integers up to 12,500, a great aid in interconverting these two scales. Candler (1951) has proposed calling the waves per centimeter unit the Rydberg. Meggers (1951) recommends calling this unit the kayser, written as K; this recommendation is supported by the Joint Commission for Spectroscopy, as reported in the *Journal of the Optical Society of America* **43,** 411, 1953. I frankly prefer primary to honorarium units, and so shall use cm^{-1} or waves per centimeter in this chapter.

There is still no standard designation of spectral axes. In general, wave length increases from left to right and wave number from right to left, but different authors use one or the other of these as the linear scale; and earlier work was often published with an abscissa linear in prism or Littrow mirror rotation angle. Theoretically absorption band shape is symmetrical with respect to energy, or on a linear wave number scale, although commonly this mirror plane symmetry is reduced or destroyed by the overlapping of other absorption bands. Ordinates may be in units of detector-recorder deflection, transmittance, absorbance, or, rarely, absorptivity, and with logarithmic or linear scales directed up or down. Ideally, spectra should be presented with some indication as to the experimental accuracy of determination of all parts. For example, on a grid whose ordinate is linear in absorbance, curve shape is in doubt over a much greater region of the paper for absorbances above 1.5 than for absorbances near 0.3; on a transmittance scale the region of possible error is more uniform over the different parts of the scale. In determining absorptivities, at the present time instrument errors, and variations among instruments, may be larger than sampling errors. For reasons such as these, Van Zandt Williams (1951b) recommends the presentation of spectra on grids with an abscissa linear in wave number, decreasing from left to right, but with different scales in different regions to minimize the waste of " empty spectral space," and with an ordinate linear in transmittance but labelled in absorbance. Because of these wide differences, there must be a very careful labelling of spectrogram axes, and optical constants should always be given with their units.

4. Sources of Information

The bibliography at the end of this chapter makes reference to a number of books on molecular structure theory and infrared techniques, including a number of physical chemistry texts with chapters on infrared methods. Review bibliographies and evaluations of infrared methods have appeared in the January issues of *Analytical Chemistry* since 1949; they are also available in reprint form entitled *Annual Reviews of Analytical Chemistry*, and in the new journal *Annual Reviews of Physical Chemistry*, since 1950. The former reviews in particular have been of great help to me, for they are systematic and wide in their coverage. The journal *Applied Spectroscopy* contains in each issue a bibliography index supplied by the Bell Telephone Laboratories which includes infrared work. Infrared techniques have notably developed as tools; there is no major "infrared" journal, in which all aspects are reported. The *Journal of the Optical Society of America* is particularly good for instrumentation articles, *Analytical Chemistry* for applications, and the *Journal of Chemical Physics* for theoretical aspects. Fortunately we are at the threshold of the use of new methods of research reporting and systematic literature coverage. There has been in operation for the past two years a program of systematic literature coverage of papers concerning infrared methods by a group of interested participants, each following a journal of his interest. Abstracts of these articles are sent to Mr. Carol Creitz, at the National Bureau of Standards. They are subsequently published on keysort cards by the National Research Council Committee for Spectral Absorption Data, with suitable punches for subject categories. The spectra are abstracted and sent to Dr. L. E. Kuentzel, of the Wyandotte Chemicals Corporation, Wyandotte, Michigan, where they are punched into IBM cards, which are subsequently distributed. Dr. Kuentzel is also developing an IBM bibliography card; before too long it will be possible to ask this IBM card file memory and its sorting machine, for example, for all references on polarized infrared spectra of proteins, and to receive the set of references in minutes.

A number of short summer courses on infrared techniques are given, and could be taken by biology students. For example, there is one at the Massachusetts Institute of Technology under the direction of Dr. Richard Lord, and one at Fisk University under the direction of Dr. Nelson Fuson. Two important United States conferences are the Symposia on Molecular Structure and Spectroscopy, of which the eighth was held in the early summer of 1953, and the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, held in the spring, which covers many techniques but is well attended by "infrareders." Publications of the proceedings of the International Conference on Molecular Spectroscopy, of which the fifth was held in Paris in June, 1953, and of the Discussions of the Faraday Society (England) on Spectroscopy and Molecular Structure, are of value.

Prospective users of infrared techniques should obtain the detailed instrument descriptions from the commercial manufacturers; these contain a wealth of basic information useful even if the instruments are not all purchased. The free periodicals, Baird Associates *Better Analysis*, the *Beckman Bulletin*, and the Perkin-Elmer *Instrument News*, often supply helpful leads. These companies are very cooperative in consultations and in trying out new methods for prospective customers. They have held a number of regional conferences for users of their equipment.

I must unfortunately admit that I have no acquaintance with the infrared work of the eastern half of the world.

II. Instrumentation and Techniques

Infrared spectrophotometers extend the sensitivities of humans to allow a consideration of finer changes of infrared frequencies and intensities than with our unaided senses. The data they provide, most commonly now in the form of automatically recorded spectra, are good to the extent that they are useful; requirements as to "goodness" differ considerably as to applications. A "good and detailed" high-resolution spectrum of a gas mixture may take too long to record and so not be as useful as a "good and fast" even though less-detailed spectrum of a reacting gas mixture. A spectrum may be good with respect to the instrument, having the absorption bands recorded at their correct frequencies and intensities, without interference of recorded misinformation, called "noise," with good resolution even of "fine structure," small bands close together, and with rapid recording. And a spectrum may be good with respect to the sample in its preparation and thickness, with the absorption bands clearly distinguished, not broadened and obscurbed by sample scattering effects, with the transmittance minima in the accurately recorded range of 20 to 60 %, and with the sample of accurately known thickness and concentration or distribution. This may require several spectra, with the sample in different thicknesses. Ideally an instrument should present us with all possible information in a minimum time from which we can select that which is useful. However infrared instruments may still set disturbing limitations for some applications, although there have been tremendous advances in the last ten years. Twenty years ago it was common for infrared spectra to be recorded by hand from galvanometer swings, wave length by wave length, often in a sub-basement during the wee and ideally vibrationless hours of the morning. Many universities did no infrared work. Since that time improvements and applications have occurred notably in detector sensitivity and especially speed, in amplifier design and stability, and in means of recording. Theoretical and empirical applications have been extended. Now a graduate physics or especially a chemistry department is lacking a tool of major use if infrared equipment is not available. The development of commercial infrared recording spectrophotometers in the last ten years has greatly aided this change, so that a biologist can now consider doing infrared work without fear of being swamped in instrumental difficulties. The instrument companies maintain excellent service field engineers, so that the biologist needs only to know the functions of the instrument controls and the criteria which indicate their proper functioning; the emphasis of his work can be on the utilization of the data. Infrared recording spectrophotometers are expensive, of the order of $15,000 to $25,000 with accessories; in universities the equipment is often shared by several departments and is operated by trained technicians. It is strongly recommended however that the biologist learn or at least see the operating and particularly the calibrating procedures of the instrument so that he may appreciate instrumental limitations. A helpful review of infrared instrumentation and techniques is that of Williams (1948).

1. Instrument Components and Adjustments

a. Sources

Infrared electromagnetic radiation is usually obtained as thermal radiation from hot bodies, although discontinuous arcs such as the low pressure mercury arc, with strong emission bands at particular wave lengths, may be used for calibration purposes. The radiation of course must be transmitted by any source envelope, or windows in this. Thus a tungsten filament lamp in glass is not suitable beyond about 2.5 μ and in quartz beyond about 4 μ, although these are intense and stable sources for work at these shorter wave lengths because of the high temperatures, to 3300°K, possible for the filaments. Inadequate work has been done with filament lamps in envelopes with salt windows which transmit the infrared radiations; a difficulty is in maintaining the salt to envelope seal at high temperatures, although a glyptal seal has been used (Smith, 1942) in another application. Moreover, there is a decreasing emissivity of tungsten and nichrome at longer wave lengths. Taylor *et al.* (1951) avoid this difficulty by using a V-shaped tungsten ribbon heated electrically to 2900°K. Nernst glowers, of rare earth oxides, and Globars, of carborundum, are the two most common infrared sources used, for they may be operated at 2000 or 1400°K respectively without envelopes. The latter is of larger size, to 4 inches long and ¼ of an inch in diameter, and is usually used in a water cooled housing. Carbon arcs can operate to 5000°K, but the wandering of the arc is a difficulty. Rupert and Strong (1950) used only the center portion of the positive crater of a large arc at 3900°K which required water cooling and a flue. Jaffe (1953) uses a smaller microscope illuminator carbon arc with a constant current power supply, and reports intensities five to ten times those of the Globar in the NaCl spectral region. However, electrodes must be changed every few hours, whereas the other sources need no attention for hundreds of hours. Hall and Nester (1952) have used a zirconium concentrated arc infrared source which they recommend particularly for microspectrophotometry. Beyond 50 μ the Welsbach mantle and the high pressure mercury arc in quartz have been used.

These sources approximate a grey- or black-body emission, as described by Planck's energy distribution law (see Forsythe, 1937), with a very rapid decrease in intensity as wave length is increased, usually compensated for by opening the slits of the monochromator. Intensities of different sources increase approximately in exponential relation to their temperatures, but the emission maxima shift to shorter wave lengths, as described by the Wien displacement law: the wave length of maximum emission times the temperature of the grey- or black-body source is a constant, 2884 microns °K. (Using this relation, the approximate temperature of a source may be determined by measuring the wave length of maximum emission using constant slit widths and single beam recording.) These radiations of shorter wave lengths are scattered more than those of longer wave lengths by imperfections in the optics, dust, etc., and hence they may appear, by extraneous paths, at the exit slit of the monochromator set for longer wave lengths, a complication which reduces the advantage of the very high temperature sources. However with the development of filters and gratings (White, 1947) and multipass monochromators (Walsh, 1952) which greatly reduce this scattered radiation, an increased use of higher temperature sources may be expected.

Source power supplies usually have a voltage control which should be adjusted from week to week to maintain an approximately constant power output as observed on the meters. The Nernst glower has a negative temperature coefficient, and so requires preheating before it will start, and a ballast in its circuit once started. This preheating is conveniently done with the Perkin-Elmer Nernst glower source by an electric heating coil, mounted next to the glower, which is briefly turned on when the glower is to be started.

By radio techniques, electromagnetic radiations down to 82 μ (Glagolewa-Arkadiewa, 1924) and to 30 μ (Lewitzky, 1927) have been produced, but such sources have not yet been perfected and the greatest part of the "microwave" work is still at wave lengths longer than 1 mm.

b. Focussing and Double Beam Optics, Accessories, and Sample Positions

Radiation from the source is focussed on the entrance slits of the monochromator, and subsequently on the exit slits and detector. Salt lenses may be used, but because of the large wave length span covered by most instruments, three octaves as compared to one octave in the visible region, the refractive indices and hence the focal positions of radiations of the extreme wave lengths are too greatly separated for convenient use. Hence reflecting optics, which are achromatic, are predominantly used, with the mirror surfaces usually being aluminum, or silver or gold (see Strong, 1938). A spectrophotometer should be designed so that the most expensive single optical element, which in the infrared region is the prism, sets the limits of the effective aperture and hence resolution of the instrument. The source mirror, which focuses the radiations onto the entrance slit, should at least fill this aperture. Spherical mirrors are usually used here, in spite of the aberrations which are introduced by their use off axis. Mirror adjustments should be made very critically, utilizing instrument deflections as a guide to results; they should not be attempted casually.

There may be a variety of accessories between the source and the monochromator. The radiation may be "chopped" by a rotating shutter, to reduce "zero drift" by allowing the comparison of the detector output to a standard voltage every half cycle and to allow the utilization of ac amplification of the detector output, electronically more convenient than dc amplification. Double beam instruments automatically compare the radiation which passes through a sample to that which passes through a blank; other parts of the optics may be single or double so long as their characteristics bear a constant ratio to each other at all intensities or wave lengths used. The Baird and Perkin-Elmer double beam instruments both use a single source, monochromator, and detector. The two beams, from two parts of one source, are combined by a rotating semicircular mirror used off axis, which first transmits one beam then reflects the other into a single path through the monochromator and to the detector. Both of these instruments also utilize a wedge-shaped comb which is moved in and out of the reference beam by a servo motor to maintain a match of intensities in the two beams as the frequencies are automatically scanned; this is called the optical null method of spectrophotometry. The linearity of recording of transmittances depends on the shape of this wedge; the chart pen records the position of the wedge. To avoid end effects, the wedge is never completely removed by the servo drive from the reference beam. At the 0% transmittance reading, as when the sample beam is blocked by a card, the wedge should completely block the reference beam and the pen should record on the zero line; it may be necessary to adjust the pen and the wedge on their connecting cables. Because of the lack of driving energy when both beams are blocked, it has been recommended that this adjustment of pen and wedge positions be made using a known absorber, such as a metal plate with holes in it with a transmittance of about 20%. The wedge adjustment is rarely necessary, and requires the removing of the instrument covers. The pen may be adjusted externally. And an external adjustment of the balance of the two beams is readily made by means of adjustable shutters on the source mirrors. Daly (1953) points out that such compensating wedges are "space-sharing" devices, such that the reference and sample beam radiations do not fall on identical parts of the detector. The Unicam double beam infrared spectrophotometer utilizes a "time-sharing" adjustable star wheel rotating at high frequency to maintian the optical null balance of the reference beam. (See also Richardson *et al.*, 1953). The two beams may be electronically instead of optico-mechanically compared, by chopping the two beams at different phases so that the amplifier may discriminate between them, with their subsequent comparison. This "direct ratio" method of Savitzky and Halford (1950) is now available in the Perkin-Elmer Model 13 spectrophotometer. The Beckman IR-2T and IR-3 instruments record % transmittance spectra not by the double beam method but by "memory standardization," in which the reference beam intensities are wire-recorded in the first scanning of the spectrum, then the sample intensities are electronically compared to this "memory" as the spectrum is scanned a second time, and the ratios of these intensities are recorded. Williams (1951a) has compared the limitations of the memory standardization, optical null, and direct ratio methods of transmittance spectrophotometry.

The sample is usually put in front of the entrance slit of the monochromator. In the Baird and Perkin-Elmer double beam instruments the sample must be put here in the two beam region, but in the Beckman memory balance instruments the sample may be put anywhere along the single beam path. Although very generally not important, sample heating effects may be minimized by putting the sample after the exit slit of the monochromator, although the optical properties of the sample and cell then critically affect the focussing of the radiation onto the detector. Any fluroescent emmission by the sample would then be received by the detector; the amount of such infrared fluorescence has not to my knowledge been investigated, but it is expectedly slight. The effect of phosphorescent emmission with a decay constant greater than the chopping period, due to sample heating, which would be very slight with the sample at the exit slit, can be eliminated by using chopped radiation. The radiation is usually not focussed onto the sample, hence requiring sample cell areas of several square centimeters. Microcells have been developed which hold the sample nearer to the entrance slit, and so require less sample. And microscopes (see Section II 6d below) have been developed which focus the radiation onto the sample. Finally, polarizers may be put in front of the monochromator to allow the study of molecular orientation.

c. Monochromators

(For some theoretical aspects of monochromators in general, see Chapter 3 of this book, by Dr. Hiskey.) These are any of the devices which receive "polychromatic" radiations as input and provide a small wave length band of radiations as output. The different wave lengths may be separated by refraction, using prisms, the method of choice of the commercial instruments, or lenses; by diffraction, using gratings; by selective reflection, the reststrahlen method; by selective absorption, using filters; or by scattering filters, which deviate a greater proportion of short wave length than long wave length radiations from the direct beam. Golay (1949b) points out that of the hundreds of watts of radiation emitted in all directions by the source, only watts fall on the input area, and only milliwatts of all source wave lengths will pass into the monochromator for those which have entrance slits and so do not receive or "see" radiations from all of the source surface radiating even within the monochromator angular aperture. And the output energy, now a small wave length band, may be but a small fraction of a microwatt. In the infrared spectral region, where source energies and detector sensitivities are much less than in the visible or near ultraviolet regions, energy availability may set severe limitations.

Monochromators then should be considered from three aspects: 1) the proportion of the source energy of wave lengths within the output wave length band which is available as output energy, 2) the width and energy distribution characteristics of the output wave length band, which determines the resolution of the monochromator, and 3) the ease of varying or tuning the wave length of the output.

Diffraction grating instruments are of use in obtaining high resolution, to 0.01 cm^{-1} in the near infrared (Rank *et al.*, 1953b), and for wave lengths beyond 50 μ for which prisms are not yet available (Oetjen *et al.*, 1952). Grating instruments diffract energy into several orders, reducing available energy at any one exit slit. And if the input energy distribution is too broad, these different orders will overlap, requiring the use of foreprisms or filters. Moderately broad wave length bands may be obtained by means of focal isolation, in which apertures are placed at successive foci for a particular wave length focused by a series of lenses, or by the reststrahlen or residual ray method (see Strong, 1938; O'Loane, 1953), in which successive reflections from various salts and other substances maintain those radiations which are highly reflected but attenuate those which are poorly reflected. Filters are becoming available for infrared work, particularly of the interference type. In general they are not tunable, and so are most suitable for particular applications. Scattering filters (Plyler and Ball, 1952) reduce the proportion of the high energy near infrared radiations in a beam, thereby reducing the effect of monochromator scattering.

The commercially available infrared recording spectrophotometers utilize prism monochromators, although they may be adapted for use with gratings. Table I presents the approximate wave length limits of prism transmittance and suitable infrared dispersion: for these values see the Inter-

TABLE I

Infrared Refracting Optics Materials

Substance	Approximate prism transmittance limits,[a] μ	Suitable infrared dispersion, μ
Glass	0.3–2	0.7–2
Quartz	0.2–3.5	0.7–2
LiF	0.2–6	1 –6
NaF	0.2–10	6 –10
CaF_2 (fluorite)	0.2–9	5 –9
CdF_2 (windows)	0.5–10[b]	
BaF_2 (windows)	0.2–12[c]	
NaCl	0.2–16	9 –15
KCL (Sylvite)	0.4–20	15 –20
KBr	0.2–25	15 –25
AgCl (windows)	0.6–25	
KI	0.3–30	15 –25
CsBr	0.3–38[d]	18 –38[e]
TlBr-TlI ("KRS-5")	0.5–40	24 –40
CsI	0.2–54	38 –54[f]

[a] Thinner sections, for sample cell windows, have sligntly extended transmittance limits. For example, a glass microscope cover slip has a useful transmittance to almost 7 μ. Prisms, lenses, and window blanks of many of these materials may be obtained from the Harshaw Chemical Co., Cleveland 6, Ohio, from the Optovac Company, North Brookfield, Mass., or from the Schutte Optical Co., Inc., 16 Dowling Place, Rochester N. Y. "Servofrax" (arsenic trisulfide) glass, with a transmittance greater than 60% for 1-11μ for 2 mm thickness, is now available from the Servo Corporation of America, New Hyde Park, N. Y.

[b] Haendler, H. M., Wheeler, C. M., Jr., and Bernard, W. J. (1953). *J. Opt. Soc. Amer.* **43**, 215.

[c] Ballard, S. S., Combes, L. S., McCarthy, K. A. (1952). *J. Opt. Soc. Amer.* **42**, 684.

[d] Plyler, E. K., and Phelps, F. P. (1951). *J. Opt. Soc. Amer.* **41**, 209.

[e] Plyler, E. K., and Phelps, F. P. (1952). *J. Opt. Soc. Amer.* **42**, 432.

[f] Acquista, N., and Plyler, E. K. (1953). *J. Opt. Soc. Amer.* **43**, 977.

national Critical Tables, G. Joos: Optical Properties of Solids, F.I.A.T. Review of German Science, 1939-1946, Part II: The Physics of Solids (P.B. 95684), and particularly the booklet "Synthetic Optical Crystals," 1951, available from the Harshaw Chemical Company, Cleveland 6, Ohio. The resolving power, $\lambda/\Delta\lambda = -\sigma/\Delta\sigma$, of a prism instrument, or its ability to record as two two absorption bands which are close together, is directly proportional to the difference of thickness of prism traversed by the extreme rays of a beam and to the dispersion of the prism substance: the larger the prism utilized or the more passes the beam makes through one prism or several prisms in a row or the more rapid the change of refractive index with wave length of the prism material the greater the resolution. Prisms of 60° angle and 75 mm. base are commonly used. In the 3 μ region quartz has a higher dispersion than lithium fluoride, but the latter is usually used to avoid the transmittance cutoff of quartz near 3.5 μ. In the 6 μ region fluorite is preferred. Sodium chloride is best in the "fingerprint" region of 9 to 15 μ, and for much work its dispersion is adequate over the range 2 to 16 μ for which it is commonly used in the commercial instruments. Potassium bromide is good to 25 μ, then the recently developed cesium bromide to 38 μ, which has better dispersion than the mixed crystal of thallium bromide and thallium iodide, or "KRS-5," and finally cesium iodide is good to 54 μ (185 cm^{-1}), the longest wave length yet available to prism instruments. Infrared prisms may cost $200 to over $1000. Their surfaces are frequently damageable by moisture in the breath or from close fingers. They should not be polished casually. In the commercial instruments, the prisms are mounted on small tables, allowing the ready interchange of prism materials. With sodium chloride prism instruments, resolving powers of 500, or 0.02 μ at 10 μ, or 2 cm^{-1} at 1000 cm^{-1}, are now common. Before changing a prism, be sure that the other parts of the optics will pass the radiation desired, such as spectrometer cover or particularly detector windows.

Monochromator slit widths also affect resolution. Narrower slits increase resolution, but they also decrease the energy available in the output, so that the record may be obscured by noise. By allowing the detector circuit a longer averaging time, by running the spectrum at a slower speed and increasing the detector filtering, some of this noise may be eliminated, allowing a recording of this increased resolution. If broad absorption bands are being studied, high resolution is not necessary, and quantitative accuracy of recording is increased by opening the slits. The commercial instruments have separate and in some cases automatic controls of slit widths, affecting signal power and resolution, of detector filtering or time constant, affecting the recording of noise or of rapid changes of signal, and of scanning speed, affecting the time taken to record the spectrum or the time allowed the detector to accurately measure the power received at any one wave-length. All of these controls must be set, or "programmed," before a spectrum is recorded; their values should be written on the spectrum. In routine work, these controls are not frequently changed, but it is strongly recommended that their effects and the limitations they modify be understood by anyone using the instrument or the data from it; this information may be provided by a set of spectra of one sample recorded under the different programming conditions. The commercial instruments now have slit mechanisms which automatically open the slits as wave length increases, to compensate for the rapidly decreasing energy of the source at the longer wave lengths. However the initial value and the range of slit openings may be adjusted before the record is recorded. In some instruments the slit control is servoed to the reference beam intensity, to maintain a constant reference energy in spite of atmosphere or solvent absorption. In other instruments, the slit control is a mechanical or electrical cam. Slit widths of the order of 0.1 mm. at 10 μ are typically utilized, with a 50- to 100-fold variation as the spectrum is scanned. The entrance slits are generally curved to compensate for the image curvature by the prism. Slit heights from 10 to 40 mm are employed. For further discussion, see the operating instructions for the commercial instruments. Resolution from the point of view of communication theory has been considered by King and Emslie (1951).

Prism monochromators usually include an entrance slit; a collimating mirror, commonly an "off-axis" paraboloid, which is a part of a parabolic surface away from the axis whose use reduces the aberrations introduced into a beam because of its not travelling along the paraboloid axis, which sends a parallel beam of rays onto the prism; the prism, which refracts the different wave lengths, sending them in slightly different directions; commonly a Littrow mirror, a plane mirror which sends the radiations back

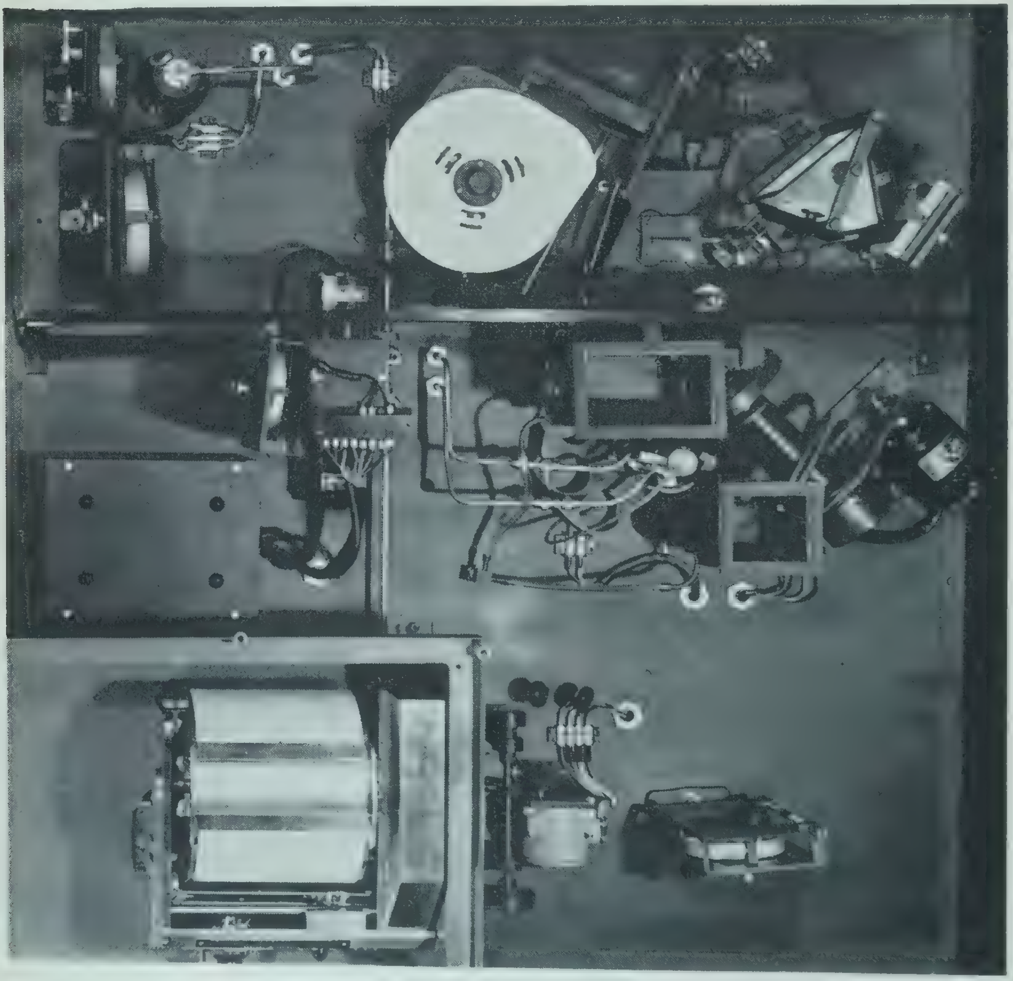


FIG. 1. Optics and mechanics of the Baird Associates infrared recording spectrophotometer. (Courtesy of Baird Associates.)

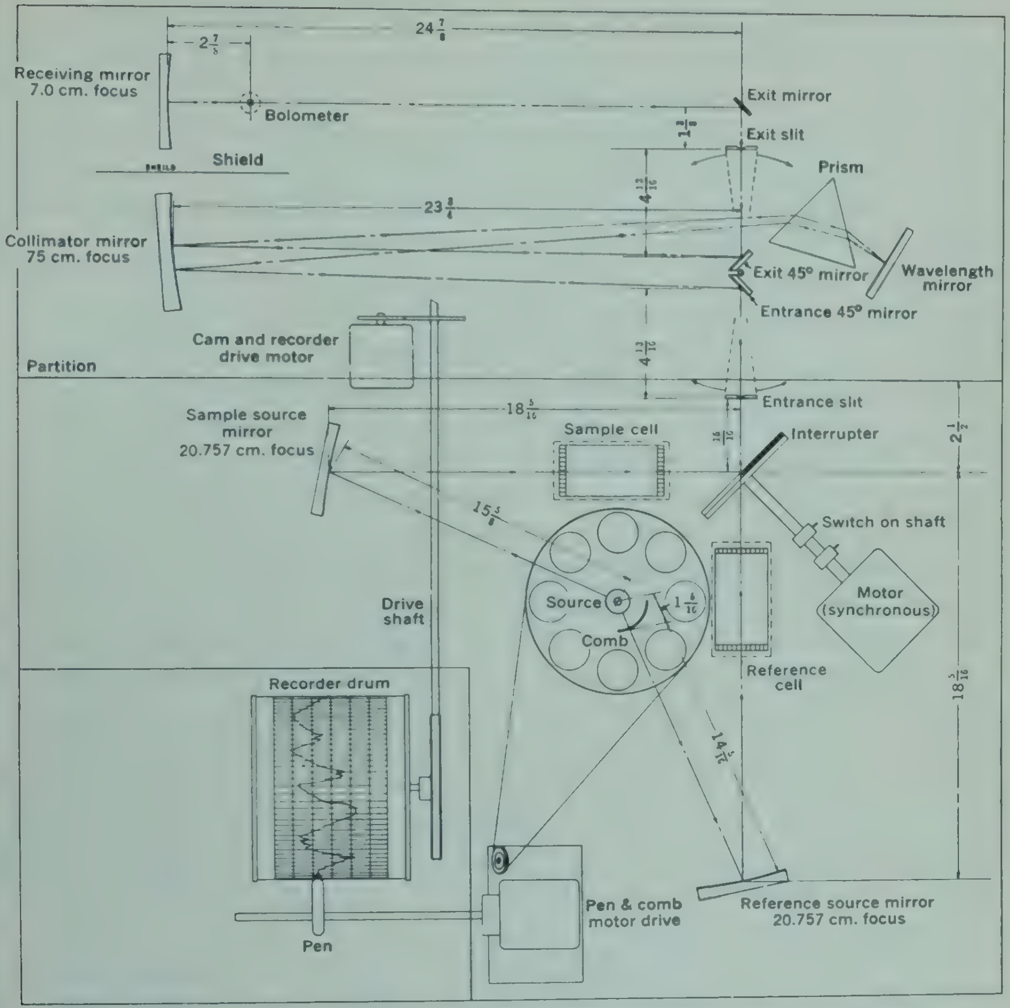

24 7/8
2 7/8
Receiving mirror
7.0 cm. focus
Bolometer
Exit mirror
1 3/8
Exit slit
Shield
Prism
4 13/16
23 3/4
Collimator mirror
75 cm. focus
Exit 45° mirror
Wavelength
mirror
Entrance 45° mirror
Cam and recorder
drive motor
Partition
4 13/16
Entrance slit
Sample source
mirror
20.757 cm. focus
18 5/16
Sample cell
Interrupter
2 1/2
15 5/8
Switch on shaft
Motor
(synchronous)
Drive
shaft
Source
1 6/16
Comb
Reference
cell
Recorder drum
14 5/16
18 5/16
Pen & comb
motor drive
Reference source mirror
20.757 cm. focus
Pen

FIG. 2. Schematic diagram of the Baird Associates infrared recording spectrophotometer. (Courtesy of Baird Associates.)

through the prism toward the collimator thereby increasing the refraction of the different wave lengths and cancelling out certain effects due to using anisotropic prism materials; and an exit slit, which passes a small part of the spectrum focussed by the collimator in the region of the exit slit. (see Figs. 1 and 2.) Monochromators may also include a number of plane mirrors, to allow the "folding" of the optics. The different wave lengths are brought past the exit slit in recording a spectrum by rotating the Littrow mirror. Monochromator alignment and focussing are very important in determining output energy; although output energy may be increased an order of magnitude or so by using a better source or a better detector, it may be improved many orders of magnitude by focussing if the monochromator has been out of adjustment.

With the source on and the room lights out, the optics should be examined. The beam should be centered horizontally and vertically on each of the optical components, and should come to a focus at the entrance slit, the exit slit, and the detector surface. Mirror adjustments should not be made without previous experience; criteria for judging their need are presented in Section II 3 below. These adjustments are made by utilizing both the visual images and the energy received by the detector, as evidenced by recorder deflections. A monochromatic source, such as a mercury arc, is of great aid in making such adjustments, particularly if it can be put in the position of the standard source. The author made such a source, to replace the Baird Globar, and found it of great convenience in this work.

A major advance in monochromator design has been presented by Walsh (1952, 1953), Ham *et al.*, (1952), and Walsh and Willis (1953). In these "multiple pass" monochromators a pair of right angled plane mirrors reflect the radiation additional times through the prism, the number of passes utilized being determined by chopping one of the reflected beams and tuning the detector output amplification to this chopping frequency. Resolutions greater than 1000 with sodium chloride optics (less than 1 cm^{-1}) as well as greatly decreased monochromator scattering have been obtained. This feature is now available in the Perkin-Elmer Model 112 monochromator. Another promising development is that of multislit spectrometry by Golay (1949a) in which the single entrance and exit slits are replaced by multiple slits, so that energies in many parts of the focussed output spectrum are utilized simultaneously by means of computer circuits. In preliminary work an effective tenfold increase in energy has been obtained.

Infrared transmitting prisms have a temperature coefficient of refractive index, dn/dt, of the order of -10^{-5} per °C; temperature variation of 1°C produces a significant shift of the spectrum unless compensated in some manner, thereby throwing off the calibration. Control of this effect is attained in two ways, either by having a bimetallic strip which with temperature change rotates the collimator mirror or the plane mirror which reflects the spectrum onto the exit slit to approximately compensate for the spectrum shift due to the change in dispersion, or by thermostating the entire instrument, by running water in the housing or by heating the instrument to a controlled temperature above that of the room. Because of this temperature effect, and because of the effects of moisture on the salt optics, it is common practice to use infrared spectrophotometers in air conditioned rooms.

The dispersion of a prism, $dn/d\lambda$, is not linear, yet for convenience of use the recorded spectra of the commercial instruments are made linear, either in wave length or wave number units, by means of cams which control the rotation of the Littrow mirror to compensate for the nonlinear dispersion. These cams of course are designed for particular prisms, so that if the prism is changed the wave length or wave number cam must also be changed to obtain linear recording, and it may also be desirable to change the slit cam. These complications are such that one does not yet change prisms in the middle of a spectrum, by means of push rods or other semiautomatic devices. For the first few times, such changes should probably be made by the instrument company's field engineer. With the present commercial instruments one can record spectra from 5000 to 625 cm^{-1} which are adequate for many purposes in about ten minutes.

Because of the longer wave length, infrared optics need not be as perfect as visible or ultraviolet optics. But all such imperfections, mirror aberrations or scratches, moisture-haze on the prism or window surfaces, and dust on the surfaces, deviate a certain amount of the radiations from their theoretical optical path. Deviations due to mirror aberrations are in general non-selective, but "scattering" deviations due to dust are greater for short wave lengths than for long. Since the source energy is much greater at short wave lengths than long, as much as 15% of the radiation passing out the exit slit when the monochromator is set near its long wave length limit may actually be of shorter wave lengths unless precautions are taken. Such monochromator scattering can be reduced to less than 0.5% by putting baffles in the optics to block these scattered rays, by using filters which reduce the proportion of short wave length energy in the beam, or by passing the radiation through more than one prism or several times through one.

Water vapor and carbon dioxide in the atmosphere may absorb up to 80% of infrared radiations passing through the instrument at certain wave lengths. (For this reason, the solar radiation which reaches the earth is very slight for wave lengths longer than 3 μ in spite of the sun's being a very high temperature source.) With double beam instruments, this absorption is ideally matched in the two beams so that it is not recorded, but in practice it is difficult to so perfectly align the double beam optics that a slight deflection of a few per cent does not occur at the CO_2 band at 4.2–4.3 μ. Commonly a dessicant is put inside the instrument cover, removing most of the water vapor, and the CO_2 bands may be removed if necessary by blowing dry nitrogen through the instrument, or by evacuating the instrument.

d. Detectors

Radiation detectors are transducers which convert electromagnetic energy into chemical energy (the eye, film), mechanical energy (the Golay pneumatic detector), or electrical energy (most other detectors). This energy is measured by a device which itself is a transducer, usually passing its information to us by visual signals. A detector may be described in terms of 1) its relative sensitivity. This is best presented in terms of a graph of output versus input radiation power. Dector "linearity" is determined from this graph. Output characteristics must be defined. These characteristics may be modified by the measuring device: there should be an overall consideration of the errors introduced in converting the radiation into the final visual information. 2) its spectral response, a graph of output versus wave length of an input of given power. It may be necessary to have families of these curves at different levels of input power. 3) its speed of response. This may be described by its time constant, the time required for the output to reach $1/e$ of its final value when the input is changed by a detectable amount. This may also be described by a graph of per cent of steady-state amplitude of output for a given constant amplitude of input variation as the frequency of input is varied. For example commercial instrument thermocouples or bolometers "follow" a square wave input variation at ten cycles per second to about 90% of the steady state amplitude. 4) its "noise" level, or the amount of misinformation in the output not directly due to the radiation "signal" input of the interest, under the various conditions of use. It is frequently useful to speak of the signal to noise ratio of a measurement. 5) its fatigue or adaptation, as evidenced by changes in the above values during use. Aspects of both 4) and 5) are included when one speaks of the "stability" of a detector. And finally 6) its other physical and chemical characteristics, and those of its surroundings, which affect its use. This would include its composition, size, particularly of its sensitive surface, perhaps its electrical resistance, effects on it of humidity and temperature changes, etc.

Photovoltaic cells are useful only to about 0.85 μ. For signalling work, infrared sensitive phosphors (O'Brien, 1946) are being developed. Photographic film sensitized out to 1.2 μ (Eastman Kodak type Z) is available. Photoemissive cells such as the cesium oxide photocell may be useful to 1.2 μ. In the visible region a photomultiplier may be 10^5 times as sensitive as a thermocouple or a bolometer., but its sensitivity decreases very rapidly in the near infrared. The infrared image converter was a war-time development, as the German "Bildwandler" or the Allied "sniperscope," in which the emitted photoelectrons are accelerated and focussed by an electrostatic lens onto a fluorescent screen. An interesting application of this for observational microscopy in the near infrared spectral region has been described by Johnson (1953). Photoconductive cells are more promising for near infrared work, with useful sensitivities of lead sulfide to 3 μ, lead selenide to 4.5 μ, and lead telluride to 5.5 μ (Simpson and Sutherland, 1952). Their noise level is decreased by using them at low temperatures (Simpson, 1948), and hence their long wave length limit is extended. Lead sulfide cells are used in motion picture sound systems; they are commercially available, as for example the "Cetron" electronic tubes of the Continental Electric Company, Geneva, Illinois, and the "Ektron" detectors of the Eastman Kodak Company, Rochester, N. Y. Their resistances are in the range 10^4 to 10^8 ohms allowing direct amplifier coupling. Their time constants are in the range of 10^{-5} to 10^{-3} seconds. They are the detectors of choice for high resolution work in the near infrared region (Rank *et al.*, 1953a). The commercial lead sulfide cells have a larger sensitive surface than is necessary for spectroscopic work; additional improvements in noise level may be expected.

The other common infrared detectors are heat detectors, whose spectral responses depend particularly on the reflectances of the radiation "receivers." The receivers are generally coated with a "black," such as platinum black, giving a flat spectral response from the ultraviolet through the infrared spectral regions. These detectors are surpassed in sensitivity by photoemissive and photoconductive cells for wave lengths shorter than about 5 μ, but for longer wave lengths in the infrared region they are still the best detectors available, although further improvements in detectors may be expected.

The Golay pneumatic detector (Golay, 1949b) has a salt window through which the radiation passes into a gas chamber within which the receiver is supported. Absorption of the radiation by the receiver causes a heating and expansion of the gas, producing a deflection of a thin limiting membrane on which there is a mirror surface. Light from an auxiliary source is reflected from this mirror onto a photocell whose output may be amplified, allowing a sensitive measurement of mirror motion and hence of the incident radiant power. A time constant of 0.001 seconds and an equivalent noise input (see below) of 5×10^{-11} watts, as measured with the operational amplifier and recorder of longer time constant, have been attained. Although the receiver of early models was larger than the exit slit image of the usual infrared monochromator, this detector is increasingly used in research instruments, and has been chosen for use in the new Unicam double beam instrument. The Golay detector rates high in the comparative study of detectors by Jones (1949). They are commercially available, with chopper and amplifier, from the Eppley Laboratory Inc., Newport, Rhode Island.

Bolometers are detectors which contain a material with a high temperature coefficient of resistance. They require an externally applied voltage, generally a battery; the varying voltage drop across the bolometer, due to the varying resistance as the incident radiant power varies, is measured by the subsequent electronics. Their relative sensitivities are expressed in microvolts output per microwatt incident radiant power, although it is a resistance, not directly a voltage, change which occurs in the bolometer due to the radiation. Bolometer performance theory is discussed by Jones (1953). The metal bolometer of the Baird instrument is a blackened platinum strip 0.2 microns thick, mounted in an evacuated chamber. The vacuum is maintained by an oil pump; with it there is a decreased conductance to the gases surrounding the detector strip and hence an increased sensitivity and improved linearity. A bolometer has also been made utilizing the resistance characteristics of the transition region to superconduction of tantalum, near 4°K (Andrews *et al.*, 1942). Although of high sensitivity (see Jones, 1949), the difficulties of controlling this low a temperature have precluded its wide use. Thermistors are bolometers which utilize the resistance properties of a semiconductor of sintered nickel, cobalt, and manganese oxides (see Wormser, 1953). Time constants of the order of 0.005 seconds have been attained. Their megohm resistance allows direct amplifier coupling. They are of rugged construction compared to other thermal detectors. They are used in a housing which is sealed but not evacuated. Their noise level is higher than that of the thermocouple or metal bolometer, preventing detection of signals below 10^{-8} to 10^{-9} watts (Wormser, 1953). Thermistor detectors with preamplifiers are available from the Servo Corporation of America, New Hyde Park, New York.

Thermocouple detectors utilize bimetallic or alloy junctions such as bismuth-antimony to bismuth-tin, or more recently of semiconductors, which have a high thermoelectric coefficient, generating a voltage proportional to the temperature differences of the junctions in the circuit. The "hot" junction is covered by a receiver, such as blackened gold leaf. The cold junctions are commonly made through the supports, which constitute a room temperature "thermal sink." In "DC" operation, in which unchopped radiation is employed, there may be a shielded junction to compensate for drifts of room temperature, but with chopped radiation this compensating thermocouple is not necessary, for only the "AC" component of the thermocouple output is amplified. To improve sensitivity and linearity, thermocouple housings are evacuated, but sealed evacuated thermocouples are available, eliminating the need for vacuum pumps. Thermocouples do not need external voltage supplies; their relative sensitivities are expressed in terms of the directly produced microvolts output per microwatt radiation input.

Metal bolometers and thermocouples are the most commonly used infrared detectors. Except that the bolometer requires an external voltage supply and the thermocouple does not, their characteristics are equivalent. Their output is linear in the operating range, with a relative sensitivity or responsivity of the order of 5 microvolts output per microwatt radiation input. In typical operation an infrared monochromator supplies of the order of 0.02 microwatts of radiation to the detector, so that its output is of the order of 0.1 microvolts, although both the input and the amplified output may be varied some thousand fold by control of slit widths or amplifier gain. These and the following parameters are all expressed as "rms" or root-mean-squared values (the square root of the mean of the squared instantaneous values) rather than peak to peak or amplitude values. The noise of these detectors, which should be measured with an amplifying system having a lower noise level but with a "band pass," or frequency sensitivity, similar to that used in the instrument, is of the order of 10^{-9} volts, so that in typical operation a signal to noise ratio of 100 is attained. Utilizing the linear response of the detector in the working range, one can calculate the power input, here 2×10^{-10} watts, which would be equivalent to this noise output. This value is referred to as the rms equivalent noise input, or rms ENI of the detector; since signal to noise ratios of 1 are barely utilizable, this value has also been called the minimum detectable signal. Jones (1952) suggested that the reciprocal of the equivalent noise input be called the "detectivity" of a detector. These detectors then have a detectivity of the order of 5×10^9 per watt; as a comparison, a photomultiplier at the wave length of its peak sensitivity in the visible region may have a detectivity of 10^{16} per watt. These detectors have time constants of the order of 0.030 seconds, short enough to allow them to "follow" the intensity changes of the chopped radiation. Their receiver areas are of the order of 1 mm^2, with a length greater than the width to receive an image of the exit slit of the monochromator, which is focussed onto the receiver commonly by an ellipsoid mirror, with about a six to one reduction in size. Accurate focussing of this mirror is very critical in affecting output. The detector is commonly mounted facing this mirror "on axis," obscuring a small amount of the radiation which falls on the back of its housing. The resistances of these detectors are of the order of 5 ohms, requiring preamplifier electronics.

e. Amplifiers and Recorders

Williams (1948) has discussed the historical developments of amplifiers and recorders for infrared studies. For accurate work galvanometer readings were used well into the 1930's, an optical amplification method, requiring many hours for the accurate determination of one spectrum. Chopped radiation was utilized by Pfund in 1929 (see Hardy, 1930) to increase sensitivity by the resonance method and to reduce the effects of thermocouple drift. Improvement in electronic tubes and circuits allowed Lehrer to build an all electronic amplifier-recorder system in 1937, but such were not commercially available until after War II. Intermediate systems involved combinations of electronic and optical amplifications, often with photographic recording of the auxiliary galvanometer light deflections.

Amplifiers should not reduce the signal to noise ratio of the detector output, thereby reducing the information in the measurement (see King and Emslie, 1953). Amplifier gains of the order of 10^5 are typically used in the present commercial instruments, to amplify the detector output of the order of 10^{-7} volts to the 10 millivolts required for the commonly used commercial stripchart pen recorders, although for weaker signals, gains to 10^7 are available. Detector noise is also amplified, but it is usual practice to speak of amplifier output noise in terms of the equivalent detector output voltage, which is of the order of 10^{-9} volts for the commercial instruments, calculated by using the gain of the amplifier calibrated under operating conditions. Chopped radiation and AC amplification are now commonly used. The low resistance of bolometers and thermocouples prohibits the successful amplification of their outputs by their direct coupling to the grids of amplifier tubes. Bolometers are usually used in Wheatstone bridge circuits, with the bridge output coupled to a transformer; thermocouples may be directly connected to similar transformers. These transformers commonly have a 300 to 1 turn ratio of the secondary to primary windings, thereby increasing the detector circuit output voltage by about this amount, and greatly increasing the effective resistance, so that the transformer output can be directly connected to the grid of the first amplifier tube. Great care must be used in the design and shielding of particularly this part of the circuit, for any "pickup" of voltage fluctuations, produced by radiations from surrounding motors, power supply lines, or other circuit components, or by mechanical vibration of these components, called "microphonics," is amplified as much as the signal of interest. To this end, the transformer and first amplifier tube are generally mounted within a few inches of the detector. Successive stages of amplification are generally conductively coupled, of the resistive or capacitive types. Noise is kept low in the amplifier by operating the tubes at less than their maximum conditions, with low cathode temperatures and plate and screen grid voltages. Selected electronic tubes of a given type, of lower than average noise level, are usually employed. Likewise the amplifier power supply circuits have voltage stabilizing components to reduce the effects of external line voltage supply fluctuations.

The minimum noise of the detector, related to the particulate nature of matter and/or energy, is "white" noise, with components at all frequencies. Likewise pickup noise, as for example that due to 60 cycle line voltage variation, is generally of different frequency than that of the signal of interest, which is determined by the frequency of chopping of the radiation. Hence the amplifier may be tuned to the chopping frequency, with filter circuits very greatly reducing noise at other frequencies. To reduce the criticalness of gain settings and chopping frequencies, the tuning of the amplifier is not made too sharp, with a "band pass" of two or three cycles at the ten or thirteen cycles per second chopping frequency generally employed. The band pass of the amplifier affects the operational noise output, for it determines the amount of white noise that is passed by the amplifier. This band pass should be stated when noise levels are given. Note also that as the band pass of the amplifier is made smaller, the signal to noise ratio is increased only if a longer time is taken to measure the signal. With the circuits commonly used, with rectification of amplified output synchronous with the chopping frequency, noise which gets through the amplifier, of just slightly different frequency than the signal, appears at the rectifier output as an AC voltage at the beat frequency, which is averaged out of the signal only after a complete period of measurement (in seconds per cycle at the beat frequency). The proportion of output noise with a low beat frequency to the signal at the frequency of the chopper increases as the band pass of the amplifier is narrowed, so that noise is reduced only by taking a longer time for the measurement. This is nicely discussed with a diagram by Wilson (1952) in the chapter on instrumentation (page 103).

With the optical null method of transmittance spectrophotometry (see Section II 1b above), linearity of the amplifiier is not required, for unbalanced signals from the two beams are amplified only to adjust the optical wedge in the reference beam to a balance. In the direct ratio and memory standardization methods, linearity of the amplifier is required, adding complications.

In order to utilize the commercial strip-chart pen recorders, which require a DC input, the amplifier output must be rectified. This is accomplished by electronic or mechanical flip-flop rectifier circuits, which are ganged in frequency and phase to the chopping of the radiation. The relative phase of the chopper and rectifier may be readily adjusted after observing the rectifier output on an oscilloscope; it should be full wave pulsating DC voltage, whose magnitude in the optical null instruments depends on the amount of optical unbalance of the two beams and whose polarity depends on which beam has the greater power. This pulsating DC is then fed through a "low pass" (rectifier output) filter, commonly an RC network of adjustable time constant, to remove the pulsating component, to a commercial recorder. In this, the signal voltage passes through a 60-cycle breaker, is further amplified, finally to drive the pen motor. In optical null instruments, the reference beam wedge position is mechanically ganged to the pen position, so that the pen motor continues to drive only until the two beams are balanced. The time that the pen takes to regain this balance, and hence the overall time constant of the instrument, is affected by the closeness of coupling of pen motor rotations and wedge position. In the Perkin-Elmer Model 21 double beam instrument, simple pulley diameter changes of this coupling may be used as a means of varying the instrument time constant;

for critical work it appears that this is a better method for controlling recorded noise than the more commonly used one of varying the electrical time constant of the low pass rectifier output filter. An overall instrument time constant of several seconds is generally used, but controls are available to change this to several minutes if spectra are to be recorded at slower speeds, taking up to several hours, if narrower slits, and hence greater resolution, or an improved quantitative accuracy, with lower noise, is desired. In single beam applications, the signal voltage may be directly compared to the slide wire voltage of the recording potentiometer. Two makes of strip-chart pen recording potentiometers are generally employed: the Speedomax of the Leeds and Northrup Company, Philadelphia 44, Pa., and the Brown Electronik of the Minneapolis-Honeywell Regulator Company, Brown Instruments Division, Philadelphia 44, Pa. In some applications, the components are removed from the standard recorder cabinet. In the simplest of these applications, the recorder chart drive and the spectrometer wave length drive are independent, both motors driving linearly in time. As a means of wave length indication on the chart, a microswitch signal activated by a cam on the wave length drive may be used to control an indicator pen on the side of the chart, or less satisfactorily to add small calibration deflections to the single recording. An improvement is to have the chart drive and the wave length drive mechanically coupled from a single motor; and in some instruments the speed of this motor is automatically reduced when the pen motor begins to turn, so that detailed spectral regions are recorded more slowly than regions in which there are few absorption bands.

With these "closed servo loop" instruments there is an optimum gain for satisfactory operation. If the amplifier gain is too great, there is a recording of excessive noise, and the pen may begin to oscillate, "hunting" for the balance position. If the gain is too small, small intensity changes are not recorded, and the recording pen may move in a sluggish and stepwise manner; with optical null instruments there may indeed be "dead stops" at which the pen position offers no reliable information as to the relative powers in the two beams: this particularly occurs when a compensation sample, such as a solvent cell, has a strong absorption band. It is recommended that the gain be set so that a small amount of noise, perhaps 1 % peak-to-peak average, be recorded on the paper, as a recorded assurance of adequate sensitivity. The amplifier gain is adjusted with single beam instruments to give an adequate deflection on the recorder. With double beam optical null instruments the gain must be great enough to provide sufficient power to drive the motor which rebalances the wedge or compensating device in the reference beam when the two beams have different intensities; the precise setting may be specified for testing purposes in terms of a voltage which should appear across the field windings of the motor when the two beams are out of balance by, say, 10%. In practice this gain setting is not too critical, and is made by increasing the gain until the pen just begins to oscillate or hunt, then slightly decreasing the gain from this position. Ideally, increasing the gain does not decrease the signal to noise ratio, for the amplifier noise level when referred to the equivalent detector output should be no greater than the detector noise level. In practice however, amplifier noise does increase out of proportion at the high gain settings, hence requiring when the radiation power is low a longer time constant and a slower recording speed to record equivalent resolution. Slit widths also affect the output signal; if the slits are opened the gain should be reduced for optimum operation. Once the gain is set to obtain the necessary signal to operate the recording system, the recorded noise may be controlled by adjusting the overall time constant of the instrument, as discussed above.

If at a necessary amplifier gain the noise is greater than about 1% peak-to-peak average, its source should be located and the causes of the noise eliminated, if possible. An oscilloscope is essential in the process; with it connected to the amplifier output, its frequency of scanning may be varied, to see if the noise has any predominating frequency, such as the 60 cycles per second of the line voltage. The noise source, particularly microphonic noise, may sometimes be located by listening. Sudden "kicks" in the record may be correlated by listening with motors going on nearby or with blinks of the room lights. The oscilloscope should also be connected to the rectifier output; an occasional source of noise of instruments with mechanical rectifiers is dirt on the breaker contacts. Subsequent tests involve the replacement of circuit components; for example high detector noise may be identified by replacing the detector by a standard resistance of similar value. The bolometer battery or amplifier batteries may become noisy, and should be replaced to see the effect on noise. Tubes may become noisy, and should be replaced by the selected tubes from the instrument maker. The vibration and shielding, particularly near the preamplifier, should be examined. In quantitating these tests, known voltages at the chopping frequency are necessary; circuits to provide them are available on some instruments.

As a final method of recording, oscilloscope presentation should be mentioned. We are not yet able to put the entire spectrum on, say, an analog to the orthicon tube, which could then be scanned very rapidly. The ordinary infrared detectors are used, with the spectrum being scanned in a few seconds down to a fraction of a second by oscillating the prism or Littrow mirror; oscilloscopes with persistent image screens may be used, with the beam being horizontally deflected in synchronism with the wave length scanning. Such instruments are essential in recording rapidly changing spectra (see for example Wheatley *et al.*, 1951) but permanent records involve photographs of the screen. Perhaps future commercial instruments will have oscilloscope presentation for preliminary sample studies, then rapid pen recordings for the permanent records.

2. Commercial Infrared Transmittance Recording Spectrophotometers

Table II lists the available instruments. They have many aspects in common, as described in Section II, 1. They all have prism monochromators; the Baird instrument has the largest prism, with a base of 100 mm. However, the Beckman IR-3, Fig. 3, has a double monochromator, with two prisms of 75 mm base, giving increased resolution and decreased monochromator scattering. They are all single pass monochromators; the Perkin-Elmer Corporation supplies the Model 112 double pass instrument, but it provides single beam rather than transmittance recordings.

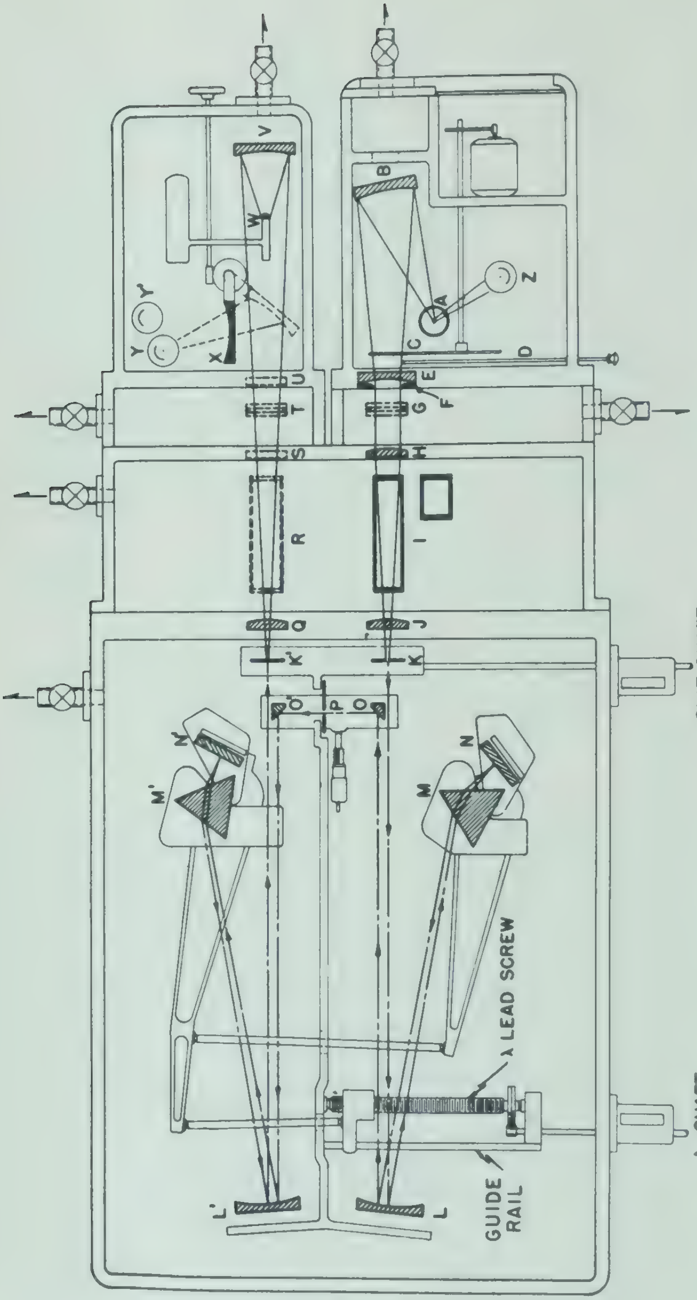
The Baird Associates (33 University Road, Cambridge 38, Mass.) Infrared Spectrophotometer (Baird *et al.*, 1947) was the first commercial transmittance recording instrument. Figure 1 shows its construction, with the mechanical slit and wave length cams in the upper center, not shown in the schematic diagram, Fig. 2. This instrument does not have the versatility of slit programming, scanning speeds, or response times of other instruments, an advantage for its use by technicians for routine work. Its standard chart, linear in transmittance and wave length, marked a big advance in the ease of comparing infrared spectra.

Beckman Instruments, Inc. (South Pasadena, California) supplies two transmittance recording instruments, the IR-2T, a modification of the earlier IR-2 single beam instrument, and the IR-3, the most expensive of the transmittance recording instruments. Figure 3 shows the optics diagram of the IR-3. Figure 4 indicates the electronic complications of the memory standardization method of transmittance recording. During the standardization (I_0) "run," the detector output is compared to an adjustable standard voltage, and a servo mechanism adjusts the slit width to provide this voltage. This slit servo motor also turns the rotor of the slit selsyn and the balance arm of an electrical cam (not indicated in Fig. 4) which adjusts the speed of the wave length motor which turns the Littrow mirrors (N and N' of Fig. 3) such that a constant, adjustable time is taken to scan through each spectral slit width. The wave length motor also turns the rotor of a selsyn. During the standardizing run the voltages of the selsyn fields, alternating at 165 cycles per second and the same for both the slit and wave length selsyns, and the voltages of the slit and wave length selsyn rotars are wire recorded by amplitude modulation of three separate carrier frequencies. During the sample (I) recording, the wire is played back (Fig. 4), and the slit and wave length servo mechanisms adjust the slit widths and

TABLE II

Commercial Infrared Transmittance Recording Spectrophotometers

Manufacturer	Source	Balance device	λ or σ Scanning	Slit control	Detector
Baird	Globar	Space sharing optical null wedge	Mechanical cam, three speeds	Mechanical cam	Bolometer
Beckman					
IR-2T IR-3	Nernst glower	Memory standardization	Optical cam, servoed constant time per spectral slit width	Servoed to constant energy during blank run	Thermocouple
	(The IR-2T has a single monochromator; the IR-3 has a double monochromator, with two prisms).				
Perkin-Elmer					
Model 21	Nernst glower	Space sharing optical null wedge	Mechanical cam, variable speeds, automatic speed suppressor	Electrical cam	Thermocouple
Model 13	Nernst glower	Direct ratio-recording electronics	No cam, variable speeds	I_0 servoed to constant energy, or electrical cam	Thermocouple
Unicam	Globar	Time sharing optical null wheel	Mechanical cam	Electrical cam	Golay


V
W
X
Y
Y'
B
A
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S
R
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Q
J
K'
K
O'
P
O
N'
M'
N
M
SLIT SHAFT
λ LEAD SCREW
λ SHAFT
GUIDE RAIL
L'
L

FIG. 3. Optical path of the Beckman IR-3 spectrometer. The source is at A and the detector is at W. (Courtesy of Beckman Instruments.)

Littrow mirror positions and the selsyn rotor positions to reproduce the conditions, and rotor voltages, of the standardizing run such that the detector output may now be directly recorded as sample transmittance. This is a "partial position" memory system, for positioning errors, due to excessive noise for example, do not accumulate until they are equal to a full rotation of the selsyn rotor. Finally, these instruments utilize optical cams to obtain linear wave length or wave number recording. The wave length servo motor, which rotates the Littrow mirrors, also moves a film, or optical cam, which has a sinusoidal density variation of slightly varying period to represent the prism dispersion, past a photocell optical system to produce voltages in a phase discriminator circuit. A standard AC voltage is produced in the selsyn field, and a servo mechanism drives the chart and selsyn rotor such that the rotor voltages are made equivalent to those of the phase discriminator. Hence the chart is driven non-linearly in time to correct for the prism dispersion and to provide a record linear in wave length or wave number. These instruments have great versatility as to control settings. It appears that a standard labelled chart is not used but that different laboratories use different scale factors, a disadvantage in comparing the spectra.

The Perkin-Elmer Corporation (Norwalk, Connecticut) supplies two transmittance recording instruments, the optical null type, Model 21 (White and Liston, 1950), shown in Fig. 5, with its optics diagram in Fig. 6, and the less expensive direct ratio recording type, Model 13 (Perkin-Elmer *Instrument News* **4**, No. 2, 1953). This company is developing the "building

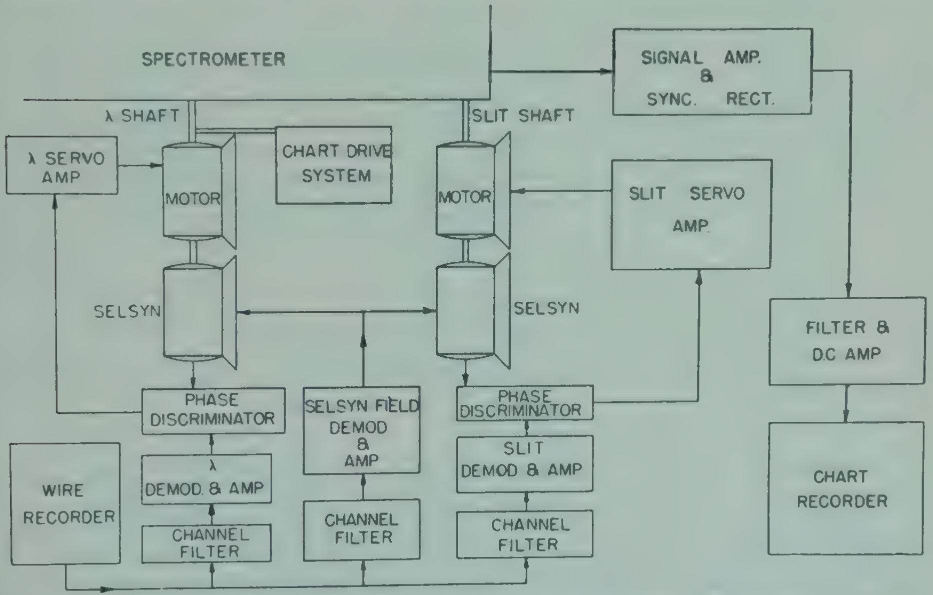

SPECTROMETER
SIGNAL AMP. & SYNC. RECT.
λ SHAFT
SLIT SHAFT
λ SERVO AMP
MOTOR
CHART DRIVE SYSTEM
MOTOR
SLIT SERVO AMP.
SELSYN
SELSYN
FILTER & D.C AMP
PHASE DISCRIMINATOR
SELSYN FIELD DEMOD & AMP
PHASE DISCRIMINATOR
λ DEMOD. & AMP
SLIT DEMOD & AMP
WIRE RECORDER
CHANNEL FILTER
CHANNEL FILTER
CHANNEL FILTER
CHART RECORDER

FIG. 4. The Beckman IR-3 recording or playback system. (Courtesy of Beckman Instruments.)

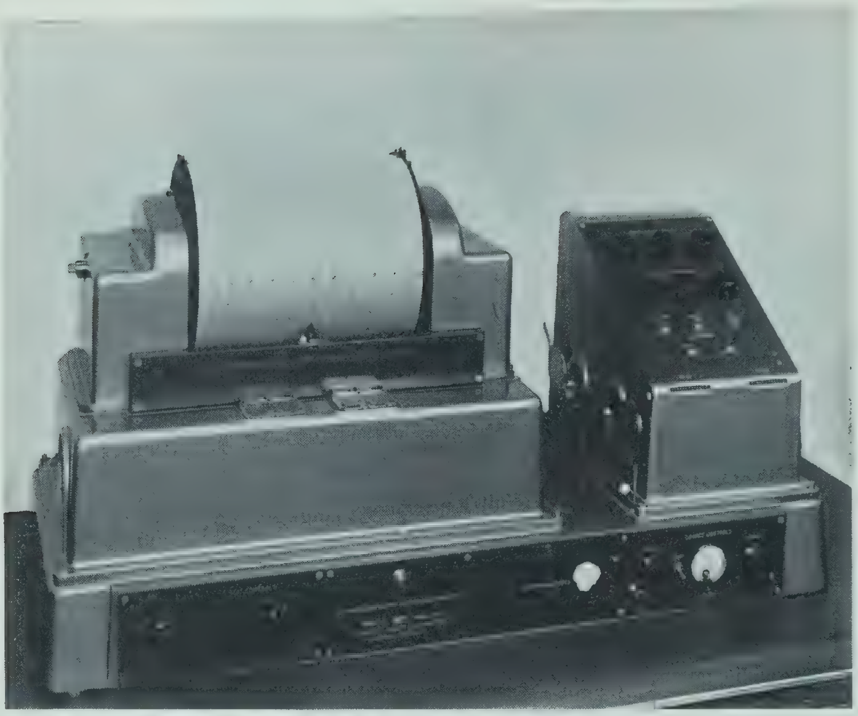


FIG. 5. The Perkin-Elmer Model 21 recording infrared spectrophotometer. (Courtesy of the Perkin-Elmer Corp.)

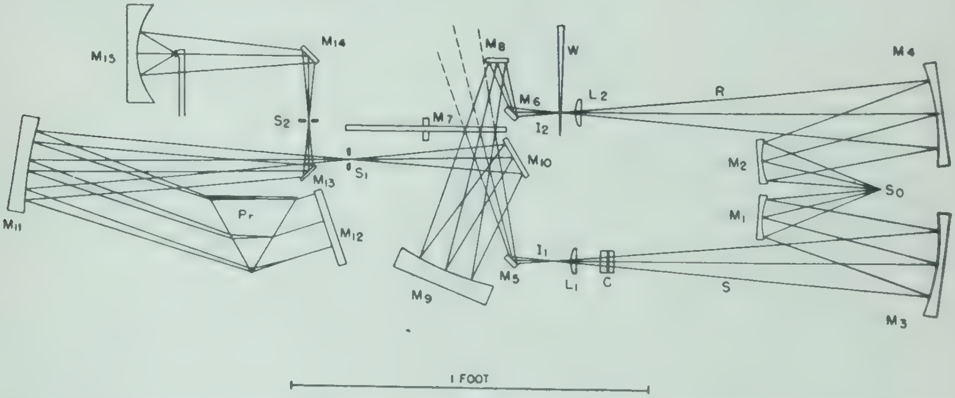

M15
M14
S2
M13
S1
M11
Pr
M12
M8
W
M6
L2
I2
M7
M10
M9
M5
I1
L1
C
M4
R
M2
So
M1
S
M3
1 FOOT

FIG. 6. Optics diagram of the Perkin-Elmer Model 21 spectrophotometer. Courtesy of the Perkin-Elmer Corp.)

block" point of view with respect to instrumentation such that these various components, sources, monochromators, detectors, amplifiers, microscopes, etc., may be readily assembled in various combinations to meet specific applications in the ultraviolet, visible, and infrared spectral regions.

Unicam Instruments (Cambridge, England) supplies a recently announced double beam instrument (see *J. Opt. Soc. Amer.* **43**, 433, 1953).

The Baird, Beckman IR-2T, and Perkin-Elmer Model 21 infrared transmittance recording spectrophotometers are competitive in price, in the range of $12,000. There is a continual improvement in component performance; it is recommended that a prospective buyer obtain equivalent calibration date from the different manufacturers as well as demonstrations of the applicability of the instruments to the specific problems contemplated.

3. Criteria of Instrument Performance: Calibration Problems and Accuracy of Data

The data provided by infrared recording instruments are two dimensional graphs, with the abscissa representing wave length or wave number and the ordinate representing "deflections," transmittances, or absorbances, although most of the published absorbance data was initially measured in transmittance or deflection. Errors in these graphs are not implicit, for they may be quite different for different instruments or techniques of use; they must be calibrated for all operating conditions of scanning speed, slit widths, filtering (affecting the overall instrument time constant), etc. Two aspects must be considered: the calibration of the errors under given conditions and the reproducibility of these errors with later use, as affected by differing room temperatures, gradually decreasing temperature of the source if unadjusted, reproducibility of positioning the paper on the recording drum, even the constancy of paper length of different batches and as affected by humidity, etc. In reading the wave number or transmittance of a recorded absorption band, "scale errors" must also be considered; the spectra should be recorded on a chart of large enough size and close enough grids so that these chart reading errors are not larger than the other errors in the spectrum up to the time of recording. Scale errors are at least of the order of magnitude of the writing width of the recording pen as read in the units of the chart; errors from changing the chart should be no larger than this. This scale error problem is a major one with respect to published spectra, for their size is reduced and many of the grid lines are left out. Publishers should be encouraged not to leave out the grid lines if the spectra are expected to have quantitative significance for others; an alternative is to publish a separate table of wave numbers and transmittance minima of the bands.

a. Wave Number (or Wave Length) Calibration

Spectra of standard substances are recorded, and the positions of the absorption bands are compared to the standard positions, which themselves were determined either by interpolation between previous standard values or by extrapolation of wave numbers by utilizing the approximately linear dispersion of grating instruments and the overlapping of successive spectral orders. The standard substances should have many sharp absorption bands, spaced throughout the spectral region of interest. The rotational fine structure of the vibrational bands of small molecules are particularly suitable; Oetjen *et al.* (1942) present 150 bands in the 5 to 15 μ region of CO_2, water vapor, and NH_3, the first two in the air through which the single beam of the instrument passed, the last in a 10 cm cell at 100–750 mm Hg pressure. Plyler and Peters (1950) give data for the mercury arc (0.54 to 2.3 μ), polystyrene (as a film 50-μ thick), and 1,2,4-trichlorobenzene, to 22 μ, with a few suitable intermediate bands of methanol, methane, methylcyclohexane, toluene, and water being listed. Randall *et al.* (1949) give the data for benzene, pyridine, and thiophene used for calibration purposes in the NaCl spectral region. In the near infrared region, didymium glass is useful. Water bands have been used out to 52 μ (Plyler and Acquista, 1953). HCl and HBr are useful in the 3 to 4-μ region. For grating instruments, bands closer together may be desired; Plyler *et al.* (1952) give 60 bands of methane and water in the 1.1 to 2.3-μ region. Downie *et al.* (1953) give a comprehensive review of substances for wave number calibration from 18,310 to 289 cm^{-1}, with detailed figures of the spectra. Cooper and Stroupe (1951), Rank *et al.* (1953a), and Heidt and Bosley (1953) discuss the interference cell method of wave number calibration. This involves determining the cell thickness, and is discussed under this heading in Section II 5e below. The present commercial instruments in general record wave lengths accurate to better than 0.02 μ; calibration is of particular importance when a new prism material without a previously designed wave number cam is being used or whenever there is any change in the optics, including even mirror adjustments, although it is strongly recommended that no data be published without a previous periodic check of the wave number calibration. When a wave number cam is not available, calibration data is expressed not in terms of an error function but in terms of the arbitrary micrometer scales or veedar counters which measure the Littrow mirror rotation; a large graph of scale reading versus wave number or wave length may be constructed. McKinney and Friedel (1948) give a mathematical equation relating these variables which gives a linear plot, perhaps allowing more accurate interpolation. Martin (1951) suggests fitting linear cords of determined equations to the calibration curve, then accurately plotting only the error functions. If the refractive indices of a prism material are known, the Littrow mirror veedar position at any wave number may be extrapolated or interpolated a micron or so by the use of the approximately linear relationship between refractive index and veedar reading. The author has found this method useful, and particularly recommends it when a new prism is used for which a wave number cam is not yet available but for which dispersion data is in the literature. If the correct wave number cam is available but optics adjustments are not yet made, it may be necessary to reposition the cam with respect to the recording drum rotation position. A convenient method of "finding" the infrared region when starting this repositioning is to utilize the energy peak of the source near 2 μ; with double beam instruments one beam is blocked, the recording drum is set at 2884/(temperature of the source in °K), or about 2 μ, and the Littrow mirror is disconnected from the cam follower and rotated by hand until the energy maximum is found, as evidenced by maximum deflections on an oscilloscope connected to the rectifier or amplifier output. With some instruments, the recorded length of the spectrum may be expanded or contracted to fit the recording paper by adjusting the length of the wave number cam follower.

b. *Slit Width and Resolution Errors*

The transmittance value recorded is an averaged value for all radiations which pass through the monochromator slits of finite width. With wide slits, sharp absorption bands are rounded off, and small bands on the sides of strong broad bands may be shifted on the wave number scale. The effective or equivalent slit width is a measure of the wave number span over which the transmittance is averaged; it is related to the resolving power of the instrument, but its precise definition requires a measurement with an instrument of much smaller slits, or more commonly an assumption, of the energy distribution, or "slit function," of the radiation which passes through the finite slits. In the infrared region, equivalent slit widths are often of the order of magnitude of absorption band widths, so that there may be considerable deviations from the absorption laws, and quantitative data from one instrument should be applied with caution to another instrument, particularly if the slit widths are different, as discussed by Philpotts *et al.* (1951). Hardy and Young (1949) (see also Forsythe, 1937, p. 182) discuss the correction of slit width errors, with possibilities for automatic devices. The equivalent slit width may be approximately represented as the wave number separation of two bands which are just resolved; the spectrum of ammonia is particularly useful in this evaluation. Emslie and King (1953) discuss the various definitions of line (band) widths and the effects on these widths of passing the spectrum through a monochromator-detector system.

c. *Response Time Errors*

If the spectrum is recorded too rapidly, so that the detector-recorder system does not have time to respond to changes of transmittance, absorption bands are again rounded off, and displaced on the wave number scale. The instrument should be allowed time to respond to the changes of the transmittance averages which are taken over the effective slit width range; thus spectra may be recorded more rapidly if there are only broad absorption bands or if wider slits are employed. For instruments with adjustable but not automatically varied scanning speeds, spectra should be recorded at decreasing scanning speeds until their changes are no longer significant for the contemplated use. Some instruments have automatic circuits which slow down the scanning speed when the transmittance pen is activated to record a sharp band. The overall response time of an instrument may be evaluated by noting the time, or the length on the recording paper for those instruments without automatic speed suppression, that the instrument requires to drop down to the 0 energy level of recording when the sample or single beam is suddenly blocked; in terms of a time constant for this full scale deflection one measures the time to reach $1/e$ or 36.8 % after the beam is suddenly blocked. To evaluate the effects of response time adjustments, of the rectifier output filter or of the mechanical coupling to the recording pen, a series of spectra of ammonia, for example, are recorded at the different settings, with measurements of the overall response time being made at each setting. Full scale response times are commonly adjustable from a few seconds to one or two minutes. In general, when the response time is increased the scanning speed should be decreased to maintain recorded resolution; the time taken to record a spectral region should be approximately the full scale time constant multiplied by the number of times the equivalent slit width may be divided into the width of the spectral region multiplied by 1 to 0.1, with the lower number being used when samples with but a few broad absorption bands are being recorded.

d. Noise Errors

Strictly speaking, noise is any aspect of misinformation in the recorded data. Some of this misinformation is of a constant nature, such as that due to the slit width error, and can be separately evaluated. Other noise is of a variable nature, and it is this, as evidenced by instability of the recording pen position, that is commonly referred to as noise. A smooth record does not necessarily have less misinformation than one with a small amount of recorded pen "wiggle," such a smooth record being obtained for example if the gain is reduced so that the pen response is sluggish, or if the time constant is very long with respect to the scanning speed; small absorption bands may be "smoothed" out of the record as well as noise. Instability of the pen position produces a minimum transmittance error, for the correct transmittance after constant factors have been considered may lie anywhere within the range of the peak to peak noise. Recorded noise may also obscure the estimation of the center of a broad absorption band, and produce an apparent shift in the position of a weak absorption band. In general small bands, 1 or 2 % in transmittance height, or changes of slope on the sides of larger bands, should not be relied upon for diagnostic purposes until the spectra have been rerun. Recorded noise may be referred to in terms of its peak to peak value, excluding extreme "kicks" caused for example by large motors being turned on nearby, or in terms of its rms value. If the recorded noise were a sine wave, the rms value would be $\sqrt{2}/4$, or about one-third of the peak to peak value. Actual recorded noise appears to have sharper peaks than a sine wave; White and Liston (1950) use one-fifth of the peak to peak value as rms noise.

e. Monochromator Scattering Errors

A certain amount of radiation will not follow the designed path through the monochromator, being deviated by mirror and prism aberrations and by dust and other damage on optical surfaces. If this radiation has been chopped, it may reach the detector indistinguishable from the radiation which has followed the normal path, so that the recorded transmittance represents an average at the designated wave number and at all other wave numbers scattered into the normal beam in the monochromator weighted in proportion to the intensities of these radiations. Ideally monochromator scattering would be measured by a filter which absorbs all radiations over the effective slit width of the monochromator and is transparent elsewhere, but because of the rapidly decreasing source energy as wave number decreases (or wave length increases), it is particularly the scattering of the higher wave numbers when the monochromator is set for the lower that is troublesome. Hence a glass filter is adequate for measuring monochromator scattering near 625 cm^{-1} when a NaCl prism is used, and an NaCl filter near 400 cm^{-1} when a KBr prism is used; the recorded transmittances of these substances should drop to zero and remain at zero throughout the low wave number region. With older NaCl instruments, monochromator scattering sometimes exceeded 15% at 625 cm^{-1}, but the newer instruments, particularly those utilizing the double monochromator or multiple pass techniques, keep the scattering error less than 0.5%. Another method of measuring monochromator scattering is to cover the lower half of the entrance slits and the upper half of the exit slits (author unknown to me). In some cases it is possible to reduce the scattering by placing baffles in the monochromator. Opler (1950) has prepared a correction table for "Spectrometry in the Presence of Stray Radiation" in which log $(100 - k)/(T - k)$ is given for various values of T and k, in which k is per cent of monochromator scattered radiation in the full scale deflection, which radiation is assumed to be completely transmitted.

f. Linearity and Reproducibility Errors

A change of sample absorption should be recorded by a correctly read change of recording position. This may be calibrated by means of samples of known absorption, for example different concentrations of a solute under conditions such that the absorption laws are obeyed, as described in the literature. A better method is to rotate in the sample beam at high speed a circular shutter out of which a sector of known angle is cut. A small 1800 rpm motor is adequate for this. Non-linearity of "optical-null" instruments (Williams, 1951) may be attributed to imperfections of the compensating device in the reference beam, or with instruments using a "space-sharing" reference wedge to non-uniform sensitivities over the thermocouple or bolometer receiver surface (Daly, 1953). The "direct-ratio" and "memory standardization" instruments require linear amplifiers. In general, linearity errors of present instruments are less than 0.5 %, particularly in the middle of the transmittance recording range. For quantitative work, it is necessary to evaluate the errors in recording the 0 % and 100 % transmittance levels as well as those due to nonlinearity. With double beam instruments, a deviating 100 % line may be due to unmatched emissivities of the two parts of the source used for the two beams (try rotating the source), or to different scattering properties of the double beam optics, or to an inadequate optical alignment, such that intensities at different wave lengths for the two beams are being compared although the two beams are supposedly matched, as evidenced by water vapor bands or particularly the carbon dioxide bands near 4.2 μ being partially recorded. The 0 % level must be corrected for monochromator scattering by measuring this scattering by a suitable filter (see above). With optical null instruments the 0 % is a "dead spot" at which the servo recording system is drifting, being cut off from optical signals. To prevent drifts below the 0 level on the chart, older instruments gave the servo system a slight electrical "uphill" drive to higher transmittances, so that it would be ready to respond as soon as signal was again available. This uphill drive was affected by the recording amplifier gain; if it was too great, water vapor and CO_2 bands appeared on the 0 % record. In the recent instruments drifts have been largely reduced; the 0 % and 100 % levels should follow the corresponding chart lines within less than 0.5 %. Absorbance reproducibility errors have been evaluated by Bowman and Tarpley (1953). In the good working range of 0.2 to 0.7 absorbance (60 to 20 % transmittance) and with care, twice the standard deviation of a set of absorbance readings of one sample on one day may be less than 0.3 %. On successive days, errors may exceed 4 %, particularly if the sample is changing. Heidt and Bosley (1953) discuss wire screens for reproducibility calibration of absorbance scales.

g. Polarization Errors

For samples with a consistent orientation across the beam area, such as single crystals, oriented fibers, etc., the transmittance may be different for different directions of polarization of the incident radiation, so that if the polarization properties of the radiation are not known, there may be errors in utilizing the transmittances of such samples. Prism instruments partially polarize the radiation which passes through them. If the deflection of the recorder when the entrance slit is irradiated with radiation whose direction of plane polarization is parallel to the slit is called I_p and the deflection with radiation polarized perpendicular to the slit is called I_s, then the polarization due to the instrument is given by $(I_p - I_s)/\frac{1}{2}(I_p + I_s)$; instrument polarization without any special polarizers in the beam may be as great as 30% (Šimon, 1951), particularly due to effects at the prism surfaces.

h. Sample Scattering Errors; Anomalous Dispersion

Some of the radiation which falls on a sample may be reflected, refracted, diffracted or absorbed and reemitted into directions other than that of the incident beam. When these aspects are not readily assigned, it is convenient to speak of the deviated radiation as having been "scattered." Ideally one would like to irradiate a sample at any wave length and measure the radiation which leaves the sample at any direction and at any wave length; actual instruments represent compromises with this ideal. A transmittance spectrophotometer, under principal discussion in this chapter, treats all scattering as absorption, unless otherwise corrected; the apparent absorption may be hundreds of times the true absorption due to the sample scattering error. It is possible to partially compensate for sample scattering, inincluding that due to reflections from the sample cell, by putting materials similar except for the selective absorption of the sample into the reference beam. Salt plates with the surfaces roughened to various degrees may be used for this. If the sample is in front of the monochromator, only that "scattering" within the aperture of the monochromator is detected; if the sample is in front of the detector, a larger proportion of the scattered radiation may be collected, particularly if the sensitive surface is of large size. Stenström and Reinhard (1926) for example used a comparison by these two methods of the apparent transmittances of skin samples for visible light to estimate the amount of scattering of skin. Another method of determining scattering is to swing the detector about the sample (Hutchins, 1898); this "goniometric spectrophotometry" (see Clark *et al.*, 1953) allows a measurement of the angular dependence of scattering necessary for both theoretical and empirical calculations concerning the scattering events.The interaction of radiation, expressed by Maxwell's equations, with spherical particles of various refractive indices and of various sizes with respect to the wave length of the radiation has been considered in detail by Mie (1908). Computations with Mie's equations are simplified by National Bureau of Standards Tables (1949) and the tables of Gumprecht and Sliepcevich

(1951). The extension of the equations to more dense media has been made by Theissing (1950). Equations derived by Debye (1947) indicated the possibility of determining particle molecular weight by measuring the intensity of the scattered radiation for solutions of macromolecules, following certain assumptions as to particle shape; this scattering method is already an important tool in the physical chemistry of macromolecules (see Chapter 2 by Oster). "Rayleigh scattering," in which the scattered intensity decreases inversely as the fourth power of the wave length, is a special case for very small particles, or for "air" molecules with diameters less than 1/1000 of the visible wave lengths. In general, the scattering observed in infrared work decreases much more slowly than $1/\lambda^4$, for the particle diameters approach or exceed the wave lengths of the radiations. For particle diameters much larger than the wave length, diffractive scattering loses its wave length dependence but refractive scattering still tends to decrease with increasing wave length due to the dispersion of the material. For particles with diameters of the order of magnitude of the wave length, there may be a scattering maximum at this wave length (see Dichtburn, 1952), producing an apparent transmission minimum on the usual infrared instrument. When the particle diameter, d, is much smaller than the wave length, scattering increases as d^3, but when d is larger than the wave length, scattering decreases as $1/d$ (see Henry, 1948). Hence when determining the infrared spectra of solids, to minimize the scattering error, use the intact material if it is optically uniform, such as a single crystal, or grind the material as finely as possible if its particle size already approaches 10 μ. Scattering rounds off the peaks of absorption bands and may obscure small bands; a series of spectra of samples ground for increasing times should be recorded until the "sharpness" of the spectrum is no longer improved. Scattering is also affected by the relative refractive index of the sample and its surrounding medium, for refractive scattering drops to zero when the two indices are alike. This "Christiansen filter effect" produces a markedly increased transmittance at the wave length at which the indices match. The wave length of this scattering minimum is shifted as the refractive index of the surrounding medium is altered, a basis for narrow band pass infrared filters (see Barnes and Bonner, 1936), and the total scattering is reduced the closer together are the dispersion curves of the sample and medium, a basis for "mulling" powder solids with mineral oil before recording their spectra (see Section II 4 below). The refractive index of a material has an abrupt fall and rise in the wave length region of an absorption band of the material, which is referred to as an "anomalous dispersion" (see Wood, 1934). If the refractive index of the sample passes through that of the medium in this region, the increased transmittance of the system partially compensates for the absorption of the sample, introducing errors in quantitative analysis, and the apparent position of the absorption band may be shifted to lower wave number (longer wave length) as much as 20 cm^{-1} (Price and Tetlow, 1948). These shifted bands may be identified by the apparent transmittance maximum, above the general "base line" absorption, which occurs on the high wave number side of the absorption band. Not all bands show this Christiansen effect: only those which involve vibrations of atoms or groups which make a major contribution to the refractive index of the sample.

i. Sample Preparation Errors

The quantitiative use of spectrophotometric data requires a knowledge of sample concentration and thickness. One should be aware that a sample may come out of solution, interact with cell walls, or decompose. A solution may warm in the beam, expanding to effectively remove some of the solute from the beam. Errors in evaluating cell thickness and the uniformity of sample distribution in the beam should be evaluated. These are discussed in Section II 5.

4. SAMPLE PREPARATION

a. Solids

These materials may be directly supported in the beam. If the crystal or fiber sizes approach the beam dimensions, polarization effects should be considered (see Sections II 3g and III 2). To obtain a single crystal, of a material which does not cleave suitable, in thin enough section for useful spectra, it may be necessary to imbed the crystal in a supporting medium, then grind it. If the particle sizes approach the infrared wave lengths, the large scattering errors (Section II 3h) may be reduced by grinding the particles to smaller size, by mortar and pestle, ball mill, supersonic vibrator (Schiedt, and Reinwein, 1952), or simply by rubbing the material between two salt plates which may have roughened surfaces. If the material is rubbery, it may be cooled with "dry-ice" (solid CO_2) or mixed with an equal volume of salt before grinding (Rippere, 1953). The material may be dissolved, then the solvent evaporated off, depositing the sample in fine dispersion on a salt plate (see Randall *et al.*, 1949). The solution may be frozen to reduce particle size even further, then the solvent dried off under vacuum (Schiedt and Reinwein, 1952). Or the material may be deposited from a suspension in a non-solvent, with particle size being roughly controlled by the layer of suspension used after time has been allowed for the settling out of the larger particles (see Barnes and Bonner, 1936). Scattering may also be reduced by grinding, or "mulling," the material (Barnes *et al.*, 1947) with a viscous liquid of similar refractive index, particularly ones with few absorption bands such as mineral oil, or "Nujol," perfluoro lube oil (E. I. duPont de Nemours and Co.), and hexachlorobutadiene (Eastman Kodak), the last two with greatly reduced interference in the 3 μ region. The viscous mull is readily spread on or between salt plates in controllable thickness. A 3-cm mortar is suitable for mulling the material, with the order of 10 mg of the sample being ground with two drops of mineral oil. In the very promising "pressed disk" method (Stimson and O'Donnell, 1952; Scheidt and Reinwein, 1952), 0.02 to 0.5% of the finely dispersed sample is mixed with powdered KBr, then pressed in a die with up to 15 tons pressure. The resulting artificial mixed crystal disk, 1- to 2-mm thick, is quite transparent and gives "sharp" spectra with a minimum of scattering errors and without solvent or suspending agent spectral interference. The sample may be melted or heated to a "plastic temperature" (see Sands and Turner, 1952) and laminated between supporting plates, or maintained in a liquid state by heating while the spectrum is being recorded (Mitzner, 1953). Some samples can be sublimed from the heated material to a cooled supporting plate (see Tyler and Ehrhardt, 1953). The degree of recrystallization in such "glasses" and sublimed films is different for different materials and may account for some spectral changes (Scott *et al.*, 1952). Finally, the spectra of solutions of solids may be recorded.

b. *Solvents*

Solids, liquids, or even gases may be dissolved in solvents before running their infrared spectra. The solution may then be handled with all the advantages of a liquid: it is optically homogeneous with particle size approaching that of molecular dispersal thereby minimizing the sample scattering errors, easily added and removed from cells of known and fixed thickness, and easily varied in thickness. A suitable solvent is one in which the sample is very soluble, one which has only a few infrared absorption bands, so as to minimally obscure those of the solute, and one of known physico-chemical characteristics, particularly as regards polarity, so that possible solute-solvent molecular interactions, which can shift the positions of the solute (and solvent) absorption bands with respect to the pure state positions, can be allowed for. Most solvents have many absorption bands (see Torkington and Thompson, 1945, also reviewed in Lecomte, 1949, p.411; and Anderson and Stewart, 1952). Hence to study all the bands of the solute it may be necessary to study solutions in various solvents. To study only one absorption band, due in large part to a particular chemical group, one solvent which does not have this chemical group may be adequate. Figure 7 presents the spectra of a number of the important solvents and mulling agents. The solubility of a substance depends on the relative attractive forces between the solvent-solvent and solvent-solute pairs of molecules or ions. If these forces are within an order of magnitude of each other or so, thermal molecular motion insures a mixing, with the solubility increasing the greater

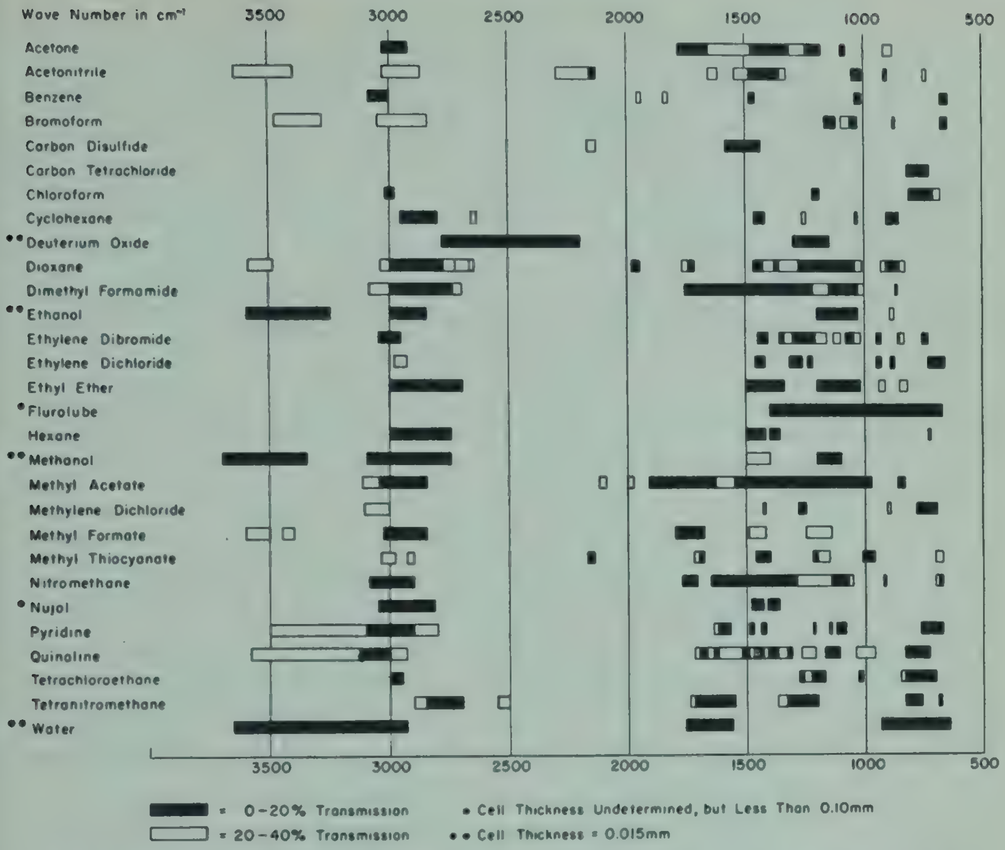
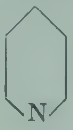
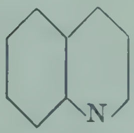
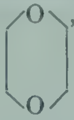


FIG. 7. Infrared spectra of solvents, for cell thickness 0.1 mm. (Courtesy of Dr. R. C. Lord, and the Spectroscopy Laboratory, Massachusetts Institute of Technology.)

the solvent-solute forces. If the solvent-solute forces are greater then the solvent-solvent forces, solvent molecules will tend to aggregate around the solute particles, and "solvation" is said to have occurred. These inter-particle forces are notably affected by the electronic configuration of the particles; if there is a net charge separation, as represented by the dipole moment of the particle, the substance is said to be polar, and interparticle forces are larger than for non-polar substances. Hence in polar solvents, polar solutes are more soluble than nonpolar; and in nonpolar solvents, nonpolar solutes are more soluble than polar. (See Chapter 10 of Syrkin and Dyatkina, 1950, for a discussion of the interrelations of molecular structures, dipole moments, and physical properties.) If in going into solution a substance is changed as to its primary bonds, as in the formation of solute ions, so that the solute particle is different from the undissolved substance, "solvolysis" is said to have occurred. This increases the solubility of a substance to the extent that the solvent-solute forces are greater than the forces between the undissolved particles. There may also be more extensive chemical reactions between the solvent and the substance to be dissolved such that the final solute consists of reaction products quite modified from the initial substance. For a further discussion, see Audrieth and Kleinberg, 1953. Table III is a list of solvents which have been used in infrared work, in sequence of decreasing dipole moment. For a number of these there are disagreements in the literature. And the accuracy of dipole moment measurements may be affected by temperature and molecular surroundings, particularly as to whether the sample is studied as a gas, as a dilute solute in a nonpolar solvent, or as the pure liquid. Hence the values in Table III should be considered as approximate. A useful reference for current work is the Digest of the Literature on Dielectrics (vol. 16, 1952, etc.), available from the National Academy of Sciences-National Research Council, Washington, D. C. Note that Table III does not necessarily indicate the sequence of degrees of solubility of a substance in these solvents, for it does not indicate the potentialities for solvation and solvolysis. Moreover, particularly as the structures become more complex, it may be necessary to consider the bond dipole moments as well as the net particle dipole moment in order to adequately visualize the oriented electric interactions between the particles. We cannot yet make adequate quantitiative predictions as to the solubilities of all substances in all solvents, but the above discussion provides the molecular point of view. Experimentation remains the usual procedure in selecting an adequate solvent for infrared studies. Carbon disulfide and carbon tetrachloride are the most commonly used. For polar solutes, the possibility of using liquid ammonia, under pressure, should be explored. The near infrared absorption of liquid water has been studied in detail by Curcio and Petty (1951); there are strong bands at 0.76, 0.97, 1.19, 1.45, and 1.94 microns. Liquid water has additional bands near 3, 4.7, and 6.2 microns, with even stronger absorption beyond 11 microns (see Plyler and Craven, 1934). Water and heavy water (D_2O) solutions have been studied by Gore *et al.* (1949), Lenormant (1952, 1953), Blout and Lenormant (1953), and others. The first authors emphasize the advantages of water solutions of various pH's in studying the structure of the ionizable groups of a substance by means of the changing infrared bands. They used cells with silver chloride windows and silver spacers 10 microns thick. They warn that the labile hydrogens of the solute may exchange with the deuterium atoms of the heavy water solvent, shifting the positions of the absorption bands of the solute. The 1640 cm^{-1} band of water is shifted to 1210 cm^{-1} for heavy water, so that the latter solvent is particularly useful for studies in the 6 micron region. Wood (1950) presents a spectrum of a living muscle fiber relative to Ringer solution. Lenormant (1953) studied 10 % protein solutions in heavy water in 30 micron cells, regulating pH by means of heavy phosphoric acid. Blout and Lenormant (1953), using a double beam instrument, were able

TABLE III

Approximate Dipole Moments of Infrared Solvents

	Dipole moment, debyes	Reference
Acetonitrile, CH_3CN	3.94	1
Nitromethane, CH_3NO_2	3.54	1
Methylthiocyanate, CH_3SCN	3.16	3
Acetone, $(CH_3)_2CO$	2.8	3
Dimethyl formamide $HC(O)N(CH_3)_2$		
Pyridine, , C_5H_5N	2.22	3
Quinoline, , C_9H_7N	2.16	3
"Heavy" water, D_2O	1.87	2
Water, H_2O	1.84	1
Methylene dichloride, CH_2Cl_2	1.78	3
Methyl acetate, $CH_3C(O)OCH_3$	1.74	3
Methyl formate, $HC(O)OCH_3$		
Ethylene dichloride, $C_2H_2Cl_2$, cis	1.74	3
trans	0	3
1,1	1.30	3
Ethanol, C_2H_5OH	1.70	1
Methanol, CH_3OH	1.69	1
2-propanol, $(CH_3)_2CH_2OH$	1.58	1
Tetrachoroethane, $C_2H_2Cl_4$, 1,1,1,2 and 1,1,2,2	1.44, 1.2	3
Ethyl ether, $C_2H_5OC_2H_5$	1.42	3
Ethylene dibromide, $C_2H_2Br_2$, cis and trans	1.22, 0	3
Chloroform, $CHCl_3$	1.15	1
Bromoform, $CHBr_3$	0.93	3
Tetranitromethane $C(NO_2)_4$	0.71	3
1,4-dioxane, , $C_4H_8O_2$	0.4	3
Carbon tetrachloride, CCl_4	0	1
Carbon disulfide, CS_2	0	1
Cyclopentane, C_5H_{10}	0	3
Benzene, C_6H_6	0	1
Methyl cyclopentane, C_6H_{12}	0	3
Cyclohexane, C_6H_{12}	0	3
Hexane, C_6H_{14}	0	3
2,2,4-trimethylpentane, C_8H_{18}	0	3

1. Syrkin and Dyatkina, 1950.
2. Gordy *et al.*, 1953.
3. Wesson, 1948.

See also Landolt-Börnstein, 1951, I. Band, 3. Teil, p. 388–505, 712–721.

to balance out most of the heavy water solvent absorption, using 25 micron cells with thallium bromide-iodide or barium fluoride windows, allowing detailed recordings of proteins, nucleic acids, nucleoproteins, and bacteria. The earlier misgivings as to the suitability of water as an infrared solvent seem now resolved. Solvent-solute interactions, with the resulting spectral changes, are discussed in Section III. With double beam and memory standardization instruments, it is possible to some extent to automatically compensate for the solvent absorption by putting pure solvent in the reference beam. This compensation is usually done empirically; if the reference and sample cells are of the same thickness, solute displacement of solvent may be sufficient to produce a greater solvent absorption in the reference cell, causing the recording pen to move above the "100%" transmittance line. This may be desirable, as an indication of increased recording error in the vicinity of these absorption bands, due to the greatly decreased signal when there is absorption in both beams or to the greatly increased slit widths used in these regions by instruments which maintain a constant I_0 signal. Perfect compensation requires using a variable thickness reference cell, set slightly less thick than the sample cell.

c. Cells

Some solids may be directly mounted in the infrared beam, and some gases may fill the entire instrument, but more commonly samples are supported on or in cells, the windows of which must transmit the infrared beam (see Table I). The sample should interact minimally with the cell windows or walls, or with the adhesive by which the windows are joined to the walls. All parts of the sample not in the beam make no contribution to the accuracy of the infrared measurement; if the sample amount is limited, cells should be only slightly larger than the beam and mounted as close as possible to a position of minimum beam size: just in front of the source housing, although this position may be too warm, or just in front of the monochromator entrance slit. If the sample amount is severely limited, some work may be done with samples or cells even smaller than the beam; a diaphragm or compensating cell of similar size is put in the reference beam. This reduces the I_0 signal, requiring increased gain, increased slit widths, a longer time constant, and a slower recording speed. The increased recorded noise sets the limit to this procedure; if very small samples are to be studied, an auxilliary beam focus position, obtained with the various infrared microscope systems, is desirable (see Section II 6d below). Solids, as mulls, melts, or sublimed films, may be supported on one salt plate, but when sample thickness measurements are to be made, or when liquids or gases are studied, two cell windows separated by a "spacer" are necessary. These cells may be either of fixed thickness, with the windows sealed to the spacer with a mercury amalgam, paraffin, glyptal, Apiezon wax, etc., or "demountable", with the windows temporarily "sealed" by pressure against a spacer such as of lead. Volatile liquids may escape too rapidly from a demountable cell; the cell should be examined after recording the infrared spectrum to make sure that no vapor bubbles have developed in the beam. Cells may be cut directly in salt (see Halford and Schaeffer, 1946). For non-polar solvents, cells as thick as one millimeter may be used, with this increasing to several centimeters in the near infrared region where absorption is much less. One-tenth-millimeter cells are commonly used, but for polar liquids cells as thin as one micron may be necessary in order for the absorption peaks to lie within the accurately measurable range of 20 to 60% transmittance. The measurement of cell thickness is discussed in Section II 5e below. "Microcells" which are mounted close to the monochromator entrance slit have been described by Wollman (1949); similar models are available from the Perkin-Elmer Corporation. Their 3-mm cell for example requires only 0.05 ml; filling is done by capillary action. Useful spectra with less than 25 micrograms of solute may be recorded. For microscope work, a capillary tube drilled in silver chloride (Blout *et al.*, 1952) allows the obtaining of useful spectra with 1 microgram of solute. In handling these or smaller samples, " manipulative losses," such as the slight deposits left on pipettes, at present limit the minimum sample amount or the quantitative accuracy which can be studied. Variable thickness cells (White, 1950; Stuart, 1953), multiple path gas cells (White, 1942), in which the radiation is reflected by mirrors at the ends of the cell to pass through the same column of gas up to 90 times, and high pressure gas cells are available from the Perkin-Elmer Corporation. Baird Associates also supply microcells and variable thickness cells. High and low temperature cells have been constructed. Neu (1953) discusses high temperature cells. Glyptal cannot be used as a sealing agent above 200°C. Dow-Corning high vacuum silicone stopcock grease serves to 350°C. Simard and Steger (1946) sealed sodium chloride windows to Pyrex spacers with a thin layer of silver chloride for a cell useful to 500°C, but thermal stresses due to the different coefficients of expansion rupture this cell if it is cooled below 150°C. With low temperature cells (see for example Carpenter and Halford (1947), Lord, *et al.*, (1952)) fogging of the windows by condensed moisture is a major difficulty, alleviated either by blowing dry gas over the cell or by having it in "storm windows," with a desiccant, dry gas, vacuum, or heating coils between the cold windows and the windows at instrument temperature. In maintaining or preparing cells, polishing the salt windows is often necessary (see Lord *et al.*, 1952). Alkali halide crystals may be cleaved using a hammer and single edge razor blade. Larger pieces may be cut with a "salt saw," a continuous silk string continuously moistened with water. Rough polishing may be done with sand paper or with #200 carborundum on a plate glass moistened with alcohol, with a micrometer being frequently used to check approximate flatness. Intermediate polishing with #600 carborundum may be useful. The author has found it convenient to do final polishing on a revolving lap made from a thick plate glass circular port-hole window mounted on a base plate and driven by a variable speed motor at 60 to 300 rpm. The axis of rotation must be accurately perpendicular to the glass surface. The glass is covered with a lintless cloth in the center of which a small amount of rouge or alumina moistened with alcohol is placed. The window, held in rubber-gloved fingers, is moved about on the repeatedly moistened center of the rotating lap until the rough grinding marks are no longer visible, then moved to the dry margin of the cloth lap and off the edge for a quick removal of the polishing compound. Holes may be drilled in salt by using a cylindrical tube with a slightly flared end which is rotated on a drill press against the salt in a moistened abrasive.

d. Liquids and Gases

Liquids may be studied as crystals at low temperature, directly as liquids or in solvents, or as gases of low partial pressure or at high temperature. Gases may be liquified or crystallized at low temperature, studied in solvents, or studied directly. Gas densities at atmospheric pressure are of the order of 1/1000 of liquid or solid densities; cells of 5 or 10 cm, or even longer length, such as the multiple path cell mentioned above, are employed. Liquids and gases may be studied in "continuous flow" cells, with the infrared data being used to automatically regulate chemical processes.

5. QUANTITATIVE ANALYSIS

The absorption, or Beer-Lambert, law may be given in the form $A = \log_{10} (1/T) = abc$, in which A is the absorbancy (previously called extinction or optical density), T is the transmittancy, a is the absorptivity of the sample in cm^2/gram (previously called the extinction or absorption coefficient or absorbancy index), b is the sample thickness in cm, and c is the sample concentration in grams/cm.[3] It is necessary to distinguish transmittance, I/I_0, in which I_0 is the power of the beam falling on the sample and I is the power which passes through the sample, from transmittancy, I/I_0', in which I_0' is the power of the beam which passes through a reference cell similar in reflectance, "scattering," and other more or less non-selective radiation losses and in the selective absorption of a solvent if used but differing in the selective absorption of the sample of interest. (Monochromator scattering is here assumed to be zero.) It is often difficult to make this perfect compensation; it is common practice to speak of the measurement as of transmittance even when there is approximate compensation, as when a roughened salt plate is used in the reference beam when recording the spectrum of a mull smeared on a salt plate. In all cases for quantitative analysis, a calibration of the adequacy of the absorption equation under the experimental conditions of measurement should be made. Barnes *et al.* (1944) illustrate a number of quantitative applications. Brady (1950) emphasizes problems of quantitative gas analyses. In the majority of quantitative studies, the height of the absorption band has been used as the means of quantitation. However, in a few studies the area under the absorption band has been used; see Section III 1.

a. Instrumental Errors

These are discussed in Section II 3, above. Instrumental errors commonly set the limits of quantitative accuracy, for signal to noise ratios over 100 for the sample beam signals, which would be about 300 for the full scale signal, are rarely employed. The effect of the finite instrument slits should be emphasized; recalibrations of the adequacy of the absorption laws, of the absorptivities, or of the empirical graphs relating the absorption variables should be made if there is any change, particularly of slit widths but also of scanning speed, gain, damping, etc., if these are markedly changed, from the initial calibration conditions. As the slits are widened, the energies which are averaged by the instrument in providing a single measurement at a given wave number setting include an increasing amount of the less strongly absorbing parts of an absorption band; the apparent absorptivities of a substance decrease if the wider slits are used, particularly if the equivalent slit widths are of the order of magnitude of the natural absorption band widths, as is commonly the case for current infrared measurements. On the other hand, the accuracy of a given transmittance or absorbance measurement is increased the wider the slit widths (or the larger the signal to noise ratio). If the purpose of a measurement is to determine the concentration of a given sample, the optimum slit width to use for this measurement is that for which the error in concentration is a minimum. This may be determined empirically by determining the errors in the analyses of a series of known concentration samples using different slit widths, but this error does not change rapidly if the absorptivities are determined for the particular slit widths used in the measurements, so that it is usual practice to use some intermediate slit width which gives a fair resolution and a fair signal to noise ratio. As illustrated by Robinson (1951), deviations from the absorption law increase as slit widths are increased. This may be visualized as follows. The radiation of wave number at the center of an absorption band is strongly absorbed. With wide slits there are also radiations in the sample beam of wave numbers away from the center of the absorption band, which are less strongly absorbed. As the beam penetrates the sample its spectral composition changes, with the radiation of wave number at the center of the absorption band being attenuated more rapidly. Hence as the beam penetrates the sample equal thicknesses of the sample absorb a decreasing amount of the remaining radiation, and hence there are deviations from the absorption laws.

b. Optimum Transmittancy

The absorbancy of a solute is given by the relation

$$A = \log [(I_0' - I_s')/(I - tI_s')] \tag{1}$$

in which I_0' is power of the radiation which passes through the reference cell which has equivalent reflection, scattering, and solvent absorption losses to the sample cell, I_s' is the power of the monochromator scattered radiation which passes through the reference cell, I is the power of the radiation which passes through the sample cell, and t is a factor which accounts for the attenuation of the monochromator scattered radiation due to its absorption by the solute. The error in A is given by

$$dA = (\delta A/\delta I_0')\, dI_0' + (\delta A/\delta I)\, dI + (\delta A/\delta I_s')\, dI_s' + (\delta A/\delta t)\, dt \tag{2}$$

$$\begin{aligned} dA = 0.4343\{dI_0'/(I_0' - I_s') - dI/(I - tI_s') + dI_s'[t/(I - tI_s') \\ - 1/(I_0' - I_s')] + dt\, [I_s'/(I - tI_s')]\} \end{aligned} \tag{3}$$

in which dI_0' is the error in the "100 %" power measurement, dI is the error in the sample beam power measurement, dI_s' is the error in the "0 %" power measurement, and dt is the change in the solute absorption of the monochromator scattering due to changes of solute concentration or thickness. As a simplifying case, if all the error is attributed to dI, and the monochromator scattering, I_s', is zero

$$dA = 0.4343\ (-dI/I). \tag{4}$$

Then the per cent error becomes

$$dA/A = -0.4343\ dI/AI. \tag{5}$$

This is rewritten as

$$dA/A = -0.4343\ dI/AI_0' \text{ exp. } (-2.303A), \tag{6}$$

in which exp. represents the number e which is taken to the exponential power given in the parentheses. This has a minimum when its derivative with respect to A is zero.

$$\begin{aligned} d\,(dA/A)/dA = 0.4343\ dI/\{A^2 I_0' \text{ exp. } (-2.303A)\} \\ - (dI/AI_0') \text{ exp. } (2.303A) \end{aligned} \tag{7}$$

On setting this equal to 0 and solving, the optimum absorbancy is found to be 0.4343, which is equivalent to an optimum transmittancy of 36.8%; the sample thickness or concentration should be adjusted to give this transmittancy. As Cole (1951) demonstrates, when all the error cannot be attributed to dI, the calculated optimum transmittancy depends on the method of combining the different errors. The optimum transmittancy also depends on the constancy of the errors in the different parts of parameter ranges. In the above derivation, it was assumed that dI was constant whatever I. If the absorptivity a of a sample and the sample cell thickness b are known to much greater accuracy, the error in a determination of a concentration, dc/c, is equal to the error in absorptivity, dA/A. Robinson (1951) has presented graphs of the error in concentration versus the per cent transmission under a variety of conditions of 100%, 0%, and sample beam power measurement errors. These curves have a broad minimum (see Fig. 8); the error in a concentration measurement is less than 4% when there is a 1% (of full scale) error of 100%, 0%, and sample beam measurements if the sample is adjusted to give a transmittancy in the range of 20 to 60%. Outside of this range, the error increases rapidly. Ayres (1949) has discussed the method of Ringbom to determine the optimum range for an analysis; make a graph of A versus $\log c$: the optimum range is that of steepest slope.

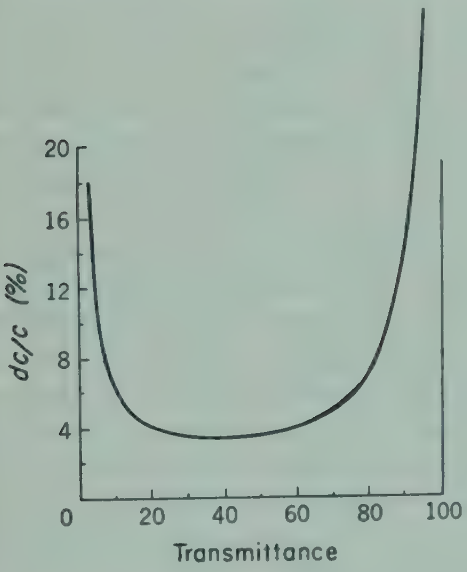


FIG. 8. Probable per cent error in composition due to probable errors of 1% in transmittance, zero, and 100% readings. (From Robinson, 1951, *Anal. Chem.* **23,** 273, courtesy of the author and the editor.)

c. Compensation Techniques

It was shown above (Eq. 3) that errors in the 100%, 0%, and sample beam power measurements all contribute to the errors of an analysis. These errors all have fixed and random components; for example the 0% error has a fixed component of the monochromator scattered radiation and a random component of the recorded noise when the sample beam is blocked. The effects of the fixed errors can be eliminated by calibration procedures, using a series of known samples which bracket in concentration those of the expected unknowns; there need only be a one-to-one, or single valued and continuous, relation between the spectra observed and the sample condition of interest. The random errors of recording set the ultimate limits of accuracy. However, it is convenient, and reduces calibration problems, if the absorbancy of the sample can be directly measured and if this absorbancy obeys the absorption laws. To do this, an instrument with negligible monochromator scattering and with compensating radiation losses in the reference beam is used. Such a measurement of the sample beam with respect to a compensated reference beam is referred to as the "cell in-cell out" technique, for in an early recording infrared spectrophotometer first the sample cell then the reference cell were automatically alternately moved into the single radiation beam (Gershinowitz and Wilson, 1938). This compensation involves the following with respect to the sample beam: 1) An equal number of reflecting surfaces. Note that the refractive indices of mineral oil and the infrared solvents are in general close to that of salt, so that their interfaces have low reflection losses. A mulled sample between two salt plates may be adequately compensated by a single salt plate. 2) Equal solvent absorption. The reference cell must be thinner than the sample cell in proportion to the displacement of the solvent by the solute. A variable thickness cell is essential for this work. The reference cell thickness is adjusted until the recorded spectrum does not deviate from the 100% line in passing through a solvent absorption band at a wave number at which the solute has no absorption. Another method is to use cells of the same thickness, but to displace solvent in the reference cell by another nonabsorbing solvent. 3) Equal scattering losses. This compensation is difficult to make perfectly; salt plates with various degrees of roughening are used. 4) Equal beam areas. This compensation is important if the sample cell is smaller than the incident sample beam. In general these compensations are not perfect, and accurate analyses can be made only over a limited range of conditions.

An alternative method of analysis is the "base line" technique of Wright (1941). Here detailed compensation attempts are not made, but the sample absorption is measured not from the 100% line but from a straight line connecting two points of maximum transmittance which are on either side of the absorption band of interest, or in the case of a solute band on the side of a solvent band the sample absorption is measured from the extrapolation of the solvent band as if the solute were not present. Unfortunately in general the base line reference points vary as the solute concentration or sample of interest is varied, so that the data may obey the absorption law only over a limited range. Tunnicliff *et al.* (1949) compare with examples the various compensation methods. Finally, if the sample absorbs strongly, the working range of accurate analyses may be extended by 1) closing the reference shutter to expand the transmittance scale, or better, putting a wire mesh in the reference beam, 2) electrically reducing the voltage produced by the reference beam, which has the same effect as 1) but without the potential optical difficulties, 3) putting a similar sample of known concentration and thickness in the reference beam, or 4) in optical null instruments replacing the regular wedge by one of smaller taper—I have used a single tooth cardboard wedge. In all of these methods, to maintain signal to noise ratio a combination of increased amplifier gain, wider slits, slower recording speeds, and increased damping must be used.

d. Deviations from the Absorption Law

Many of the deviations observed in the absorption law using the operational parameters are instrumental errors, such as errors in measuring transmittance, the noise limitation, the effects of finite slits, etc., and are not inherent in the sample, as mentioned in Hardy and Young's (1948) "defense of Beer's law." Included with these should be errors such as those due to the warming of the sample in the beam, which produces density changes which effectively removes some of the sample molecules from the beam; scattering by the sample—the ideal instrument, a goniometric spectrophotometer, would directly measure this scattering and so correct for this radiation loss; and, in part, the apparent overlapping of the absorption band of interest by other bands. Deviations from the absorption laws which can be attributed to the sample involve changes of intermolecular forces as the solute concentration is varied. A polar substance in a less polar solvent has increasing solute-solute interactions as the solute concentration is increased which interactions can change the vibrational transition probabilities and so change the absorptivities. Likewise polar solutes in solvents of varying polarities, or gases at different pressures, may have varying absorptivities. And a substance in general has different absorptivities as it changes from a gas to a liquid to a solid. These intermolecular interactions are discussed further in Section III. Lee (1946) reviews the mathematics for making corrections on the absorption law. Even these molecular interaction "deviations" may be attributed to our using the wrong parameters in the absorption equation. We should use the absorptivities and concentrations of any reaction products under the conditions of the measurement rather than those of the separated components. Hardy and Young (1948) describe a procedure for calculating the spectra of these reaction substances which does not require their isolation. Finally, the "distribution error" may be important in the quantitative study of solids. If the sample is not uniformly distributed across the beam, radiation through thin parts of the sample will have a different attenuation than that through the thick, and the averaged effect on the entire beam will not obey the absorption law. This error is of particular importance in studying tissues; see Ornstein (1952).

e. Measurement of Cell Thickness

This discussion follows Smith and Miller (1944). For cells thicker than 0.5 mm it may be adequate to measure the spacer or gasket with a micrometer before assembling the cell. For thinner cells, the thickness of the film of compound which seals the gasket to the salt windows becomes significant. For cells in the range 0.1 to 5 mm a traveling microscope may be used. The accuracy of this measurement increases the higher the numerical aperture (the less the depth of field) of the lens system. Note if an ordinary microscope is used that the fine adjustment motion is not linear but must be calibrated in the part of its motion that is used for the measurement. The area (field size) involved in this measurement is in general less than 1 mm in diameter; non-uniformities of cell thickness may be evaluated by repeated measurements. Cell thicknesses may be determined by filling the cells with substances whose absorptivities are known under the conditions, particularly as to slit widths, of the measurements. If the various cell compensations can be adequately made, so that accurate absorbancies can be measured, a single measurement is adequate, with the thickness being $b = A/ac$. A series of measurements however may be more reliable; Smith and Miller (1944) suggest using the 3.4 μ band of n-hexane in carbon tetrachloride. A graph of the measured absorbances A versus the known $(a_1c_1 + a_2c_2)$ for a series of solutions of known concentrations (c_1 is the solute, c_2 is the solvent) has a slope equal to the cell thickness. Smith and Miller (1944) discuss the interference method of determining cell thickness, which they found useful in the range of thicknesses from 0.005 to 2 mm. The beam which undergoes two reflections back and forth inside the cell can interfere minimally with beam which passes directly through the cell if its additional path length, $2b$ times the refractive index of the medium in the cell, is an integral number of wave lengths. With the cell filled with air, the amplitude of these interference bands, which cross the spectrum with transmittance maxima at each wave length which satisfies the above condition, is less than 1 %. With media of higher refractive index than air in the cell, the interference bands are negligible. The bands should be recorded under conditions of low noise. I have found it useful with the Baird instrument to add to the end of the regular reference beam compensation comb a single cardboard tooth of gradual taper; with this tooth as the compensation device, the amplitudes may be increased five times or more to allow greater reliability in reading their positions. With instruments which can make single beam records, the interference band amplitudes may be increased by increasing the amplifier gain. If there are m interference minima between λ_1 and λ_2, Smith and Miller (1944) give the cell thickness as $2b = m\lambda_1\lambda_2/(\lambda_1 + \lambda_2)$; this is equivalent in wave number measure to $2b = m/(\sigma_2 + \sigma_1)$. If sufficient fringes are obtained (the thinner cells have the fringes farther apart) the error in the thickness measurement may be less than 0.1 %. Instead of putting all the wave length or wave number dependence on the final bands, Sutherland and Willis (1945) suggest plotting the interference band number, of arbitrary origin, against the wave number of the band; if transmittance maxima only are numbered, the slope of this graph will be $2b$, whereas if maxima and minima are numbered, the slope will be $4b$. Small errors in reading the wave numbers of the bands will be averaged out. This interference cell method may also be used for wave number calibration; see Heidt and Bosley (1953). For measurement of cells of thickness less than 0.050 mm with an error less than 0.2 μ, Jaffe and Jaffe (1950) added a microscope to a Rayleigh interferometer, with measurements made with visible light.

In some quantitative applications, it may not be necessary to directly measure cell thickness. Barnes *et al.* (1947) measured the per cent penicillin in a powdered sample by adding a known proportion of DL-alanine as "internal standard." Quantitation was in terms of the ratio of the absorbance of penicillin at 703 cm^{-1} to the absorbance of alanine at 851 cm^{-1}; cell thickness cancels out of this ratio. Likewise Darmon *et al.* (1948) measured the relative amounts of leucine and isoleucine in a hydrolysate powder by a suitable absorbance ratio. It is not usually convenient to introduce solids into a cell of fixed and previously measured thickness. One may mull the sample in perfluorokerosene, and measure the cell thickness by the absorbance of the C-F band, although it is a broad band in the 8 μ region in which the sample may also absorb; perhaps the area under this band, after suitable correction for an average sample contribution, could be used.

f. Mixture Analysis

If a mixture of unknown components is to be analyzed, it is necessary first to identify the components, to evaluate how much each contributes to the observed absorption bands. This may be done by searching the catalog of known spectra at the wave numbers of the strongest observed absorption bands, particularly in the "fingerprint" region of 9 to 15 μ (1111 to 667 cm^{-1}). As each component is identified, its contributions to all the other observed bands are tabulated, and the search of the catalog is continued at the unidentified bands until all are accounted for. If there are not too many components (less than ten) and particularly if each is represented by an absorption band in the mixture to which the other components contribute minimally, the analysis may be made by infrared spectra. If there are more than perhaps ten components or if the observed absorption bands are too broad to allow a unique identification of the possible components (an extreme example would be the infrared spectrum of a tissue; see Section IV) it is necessary to fractionate the mixture before infrared analysis. This fractionation may be carried out by distillation, chromatography, countercurrent distribution, differential solubilities, fractional precipitation, specific chemical reactions followed by one of the above procedures, or any other quantitative fractionation procedure of analytical chemistry. Once the components are known, including the reaction products if any of the components interact, their proportions in a mixture may be determined by comparison of the observed mixture spectrum to a series of spectra of known mixtures even if the components do not obey the absorption law. However, it is very convenient if each of the components and their mixtures obey the absorption laws (this should be tested by experiment), so that the absorbancy of the mixture is equal to the sum of the absorbancies of the components. Equations of the form

$$A_1 = b(c_{\text{I}}a_{\text{I},1} + c_{\text{II}}a_{\text{II},1} + c_{\text{III}}a_{\text{III},1} \cdots)$$

$$A_2 = b(c_{\text{I}}a_{\text{I},2} + c_{\text{II}}a_{\text{II},2} + c_{\text{III}}a_{\text{III},2} + \cdots)$$

then apply, in which the roman numerals represent the components and the arabic numerals represent the wave numbers of the absorption bands at which the absorbancies are read. These are chosen as far as possible such that at each wave number the absorptivity, a, of one component is much larger than the absorptivities of the other components. As many of these equations are needed as there are components, and the use of additional equations, representing additional absorption bands of the mixture, may increase the accuracy of the analysis. The solutions of these linear simultaneous equations for the concentrations c may be carried out in general by use of determinants, or in particular by successive approximations. One or a pair of desk calculators aid this work, but the sequences of multiplications and summations are so tedious that more elaborate calculators are recommended. One such is the Phillips 66 Spectro Computer, Model 110 (Phillips Petroleum Company, Bartlesville, Oklahoma). This is a DC electrical analog computer into which the known parameters of the simultaneous equations are inserted on one set of potentiometers and the unknowns are determined by iterative adjustments of a second set of potentiometers using a galvanometer circuit of increasing sensitivity. Analog computers are also made by the Consolidated Engineering Corp. (Pasadena 4, Calif.) and the Computer Corp. of America (New York 7, New York), and others. The more rapid and more expensive calculators automatically make the iterative adjustments; King and Priestley (1951) discuss the use of International Business Machines (New York 22, N. Y.) punch card calculators with which calculation time may be reduced to 12 minutes per sample. In some cases only one component of a mixture is of interest; Allen and Rieman (1953) discuss experimental and mathematical shortcuts for such a calculation. It is pleasant to visualize the ideal computer. Standard data in the form of absorptivity versus wave number, determined under the operating conditions of the spectrophotometer, would be fed into the instrument for each component in the form of punch cards or tape, and converted to electronic memory, with the resolution of the spectrophotometer, for rapid reference. Hand or automatically operated circuits would allow the observation on an oscilloscope of computed spectra of these components in any concentration, mixture, and thickness. (21-inch direct view oscilloscopes are now available from the Technomatic Instrument Company, 2316 Pico Boulevard, Santa Monica, California. This large or larger size oscilloscope is recommended to reduce the "scale errors" in reading the spectra.) Trimer circuits to account for deviations from the absorption law might be available. The experimental mixture spectrum would also be observed on the oscilloscope , and the computer would automatically adjust the component proportions to closest match of the spectra. Hardy and Young (1948) discuss a similar instrument, but without the memory circuits. As examples of analyses, Kent and Beach (1947) analyzed a six component hydrocarbon mixture. Heigl *et al.* (1947) analyzed a more complex mixture, utilizing fractionation by distillation before recording the infrared spectra. Parke *et al.* (1951) determined one component in a drug mixture by its ultraviolet absorption, and separated others by their solubilities. Avery and Morrison (1947) used liquid nitrogen temperatures to "sharpen" the infrared spectra, which reduced the overlap of the bands of the components; they predicted the possible analysis of a mixture of 18 octane isomers. Such analyses may be accurate to about 1 % of the total sample, as limited by the signal to noise ratio of the measurements. Note that this would be a 100 % possible error in the actual amount of a component which is 1 % of the total sample. Repeated measurements on the same sample are recommended; statistical treatment of the repeated data reduces the probable error. Studies have been made of mixtures of adenine and guanine, (Clark, 1950a). Five per cent adenine is detectible in a mulled mixture with guanine, as added "shoulders" to the guanine absorption bands. By the wave length positions of these shoulders it is possible to say that the impurity includes adenine (note particularly the adenine bands near 9.8, 10.6, and 11.0 microns), but other impurities in less concentration might not be detected.

6. Developments in Instrumentation

a. Non-dispersive Analyzers

These instruments measure the total radiations which pass through samples and standards. They are sensitized to particular standard samples by putting these in appropriate filter cells. The types are surveyed by Patterson (1952); quantitative applications are discussed by Koppius (1951). These instruments are becoming important in automatic and continuous plant stream process control of liquids or gases, but they do not have the versatility of dispersive instruments in detecting unsuspected components in an analysis, and so are of more use in engineering (even biological engineering) than research applications. Non-dispersive analyzers are made by Baird Associates, the Perkin-Elmer Corporation, Philips Laboratories, Inc. (Irvington-on-the-Hudson, New York), Leeds and Northrup Co. (4934 Stenton Avenue, Philadelphia 44, Penna.), and the Mine Safety Appliances Company (Braddock, Thomas, and Meade Streets, Pittsburgh 8, Penna.). "Monochromatic" infrared filters are being developed, particularly of the interference type, and these will be of use in specific applications, but it is suspected that inexpensive infrared filter photometers cannot have the wide use of present colorimeters because of the extensive overlapping of infrared spectra of different components.

b. *Emission and Reflection Studies*

In emission studies, the sample, gas, liquid, or solid, thermally or electrically (Talley *et al.*, 1952) excited, replaces the usual infrared source, and the spectrophotometer is used in a "single beam" manner. The emission should be uniform or reproducibly regulated throughout the time required to scan the spectrum. For quantitative studies, it is necessary to know the aperture and effective slit widths of the monochromator, and the transducer constant (watts radiation input per unit recorder deflection output, calibrated by using a standard source) of the detector system. For qualitative studies of emission band positions, it may be adequate to use constant slit widths and simply record "deflections" versus wave length or wave number. As examples of studies, Plyler and Humphreys (1948) describe the emission of hydrogen and natural gas flames, with many bands attributed to CO_2, H_2O, OH, and CH_4. Lee (1953) notes further studies. Bullock and Silverman (1953) recorded on a twin beam oscilloscope pressure and single-wave length emission changes of a confined explosion, with a time resolution of the data of the order of 10^{-3} seconds. Hardy and Muschenheim (1934) studied the emission from humans. Reflection studies require accessory optics (see for example Oldham, 1951). They are particularly useful for highly absorbing substances, as discussed by Šimon (1951). Diffuse reflectance may be measured by using a reflecting hemisphere (Derksen and Monahan, 1952) or studied in more detail by means of a recording goniometer spectrophotometer (Clark *et al.*, 1953). A reflectance attachment is available from the Perkin-Elmer Corporation.

c. *Polarization Accessories*

Near infrared dichroic absorption polarizers, to 2.5 μ, are available from the Polaroid Corporation (Cambridge 39, Mass.). Transmission polarizers (see Newman and Halford, 1948), in which a series of plates are set at the Brewster angle to the incident radiation for most efficient isolation of a polarized component, are available from the infrared instrument manufacturers. They are commonly made with AgCl plates, but ThBr-ThI plates (Lagemann and Miller, 1951) and selenium films (as transmission or reflection polarizers, Elliott *et al.*, 1949) have also been used. For the wave length range 100-500 μ a grating polarizer has been made (Mertz, 1953). Applications with polarized radiation are discussed in Section III.

d. *Microscopy*

As mentioned in Section II 1b, spectra of small samples may be obtained by using auxiliary optics, focussing the radiation onto the sample then forming an enlarged image of the sample on the entrance slit of the monochromator. Anderson and Miller (1953) use a pair of plano-convex silver chloride lenses with the sample placed in between. There was a three-fold reduction of beam size at the sample; 20 to 50 microgram samples could be studied, as compared to the 10 milligrams or more required for ordinary spectra. With lens systems, different wave lengths come to a focus at slightly different positions. This "chromatic" aberration can introduce serious errors in the transmittance measurements at different wave lengths, particularly for higher magnification lens systems, if the lenses are not refocussed at the different wave lengths. This difficulty is avoided by using mirror optics. Elliott *et al.* (1950) used a pair of off-axis paraboloids, which were made by controlled evaporation of selenium onto spherical mirrors, with a threefold magnification of the sample image at the entrance slit. Barer *et al.* (1949) were the first to use reflecting microscope optics (see Fig. 10) for infrared spectroscopy. They had Burch aspherical mirrors and obtained useful spectra with a calculated 10^{-7} to 10^{-8} grams of sample in the field. They present spectra of frog nerve and muscle, and suggest the possibility of following cellular processes with a cathode ray tube infrared recorder. Gore (1949) used the Bausch and Lomb spherical mirror reflecting optics, to obtain useful spectra with crystals weighing about three micrograms. Wood (1950) describes the construction of the Burch type aspheric reflecting optics of 0.6 numerical aperture and 65× magnification, and presents the spectrum of a single frog muscle fiber, using monochromator slits about three times as wide as those required for "macro" spectra. Blout *et al.* (1950) and Blout and Bird (1951) discuss quantitative aspects of the problems of infrared microspectroscopy. It is the signal to noise ratio of current instruments which limits the minimum sample size which can be studied. In order to distinguish the sample spectrum from the background noise the sample must have appreciable thickness, often over 25 microns. All the radiation which passes through the sample is not received by the monochromator if the magnification of the microscope is greater than the ratio of the numerical aperture (N.A.) of the objective to that of the monochromator; magnifications greater than about 10× can be used only with a loss in the signal to noise ratio, which loss must be compensated if useful spectra are to be obtained by using wider monochromator slits, or slower scanning speeds with longer time constants (see Section II,1, above). These authors used sample areas greater than 50 by 600 microns, with sample weights in the microgram range. They emphasize that quantitative studies of smaller samples are limited more by manipulative losses of the sample, in pipettes etc., than by spectrophotometric errors. To obtain useful spectra of tissues, some 500 cells had to be in the infrared beam. (Single large cells, such as muscle and nerve may however be studied.) Fraser (1950) recognizes similar limitations. Barer (1950) emphasizes biological aspects. The minimum resolvable sample distance of an optical system without aber-

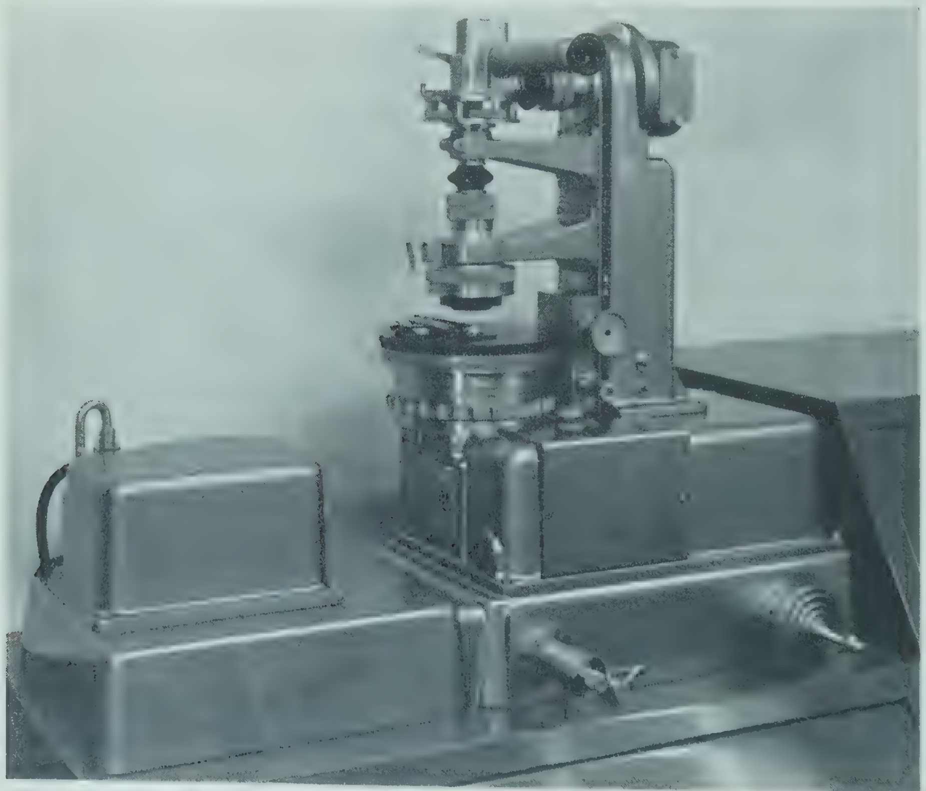


FIG. 9. The Perkin-Elmer infrared microscope. (Courtesy of the Perkin Elmer Corp.)

rations and with a detector system of higher resolving power is 0.6λ/N.A., which for most biological cells is commensurate with total cell dimensions for wave lengths in the infrared region. Quantitative measurements require sample dimensions greater than 3λ (Caspersson, 1936, reviewed in 1950. This is also discussed by Grey, 1952, in terms of the Airy diffraction pattern.) Hence it appears that we cannot expect to measure spectral differences of intact cell components, except for unusually large cells, even if there is a great improvement in instrument signal to noise ratios. Tissue spectra are discussed in Section IV below. Cole and Jones (1952) discuss another infrared microscope. A commercial infrared microscope, Fig. 9, is now available from the Perkin-Elmer Corporation (Coates *et al.*, 1953). This instrument is put at the exit slit of a monochromator. Sample areas less than 650 by 220 microns may be studied by adjusting a rectangular

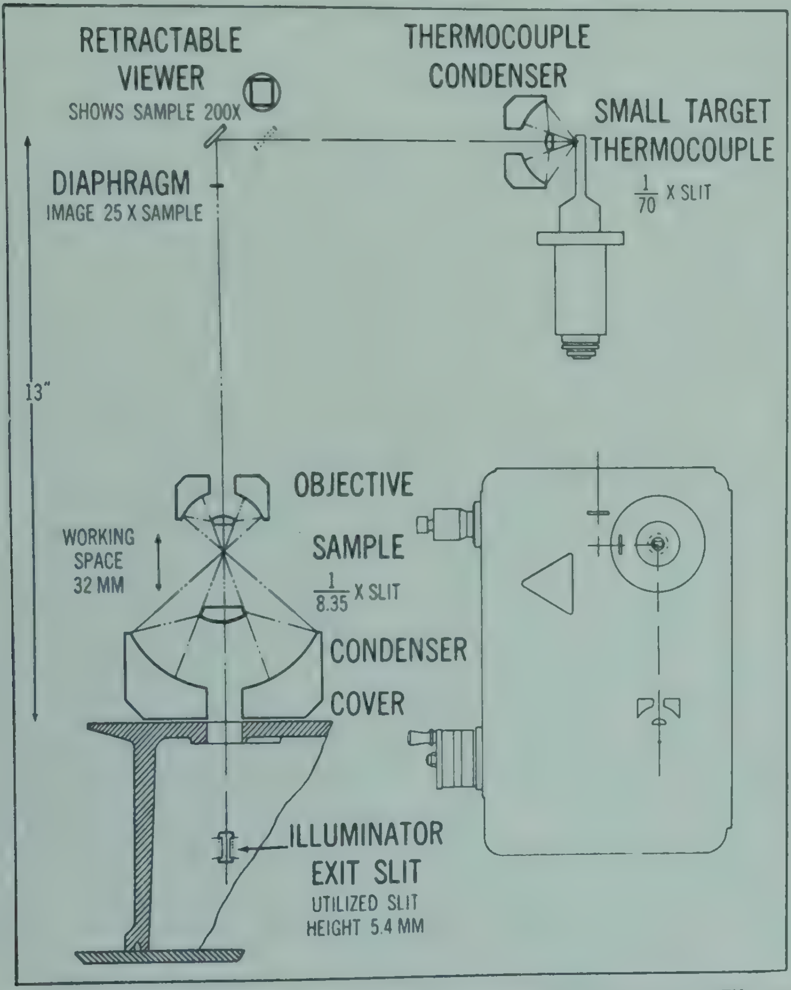

RETRACTABLE VIEWER
SHOWS SAMPLE 200X
THERMOCOUPLE CONDENSER
SMALL TARGET THERMOCOUPLE
$\frac{1}{70}$ X SLIT
DIAPHRAGM
IMAGE 25 X SAMPLE
13"
OBJECTIVE
WORKING SPACE 32 MM
SAMPLE
$\frac{1}{8.35}$ X SLIT
CONDENSER
COVER
ILLUMINATOR
EXIT SLIT
UTILIZED SLIT HEIGHT 5.4 MM

FIG. 10. Schematic diagram and specifications of the Perkin-Elmer infrared microscope. (Courtesy of the Perkin-Elmer Corp.)

diaphragm, viewed and centered by auxiliary optics at a magnification of 175×, but in general one dimension of the sample must be more than 100 microns in order to obtain useful spectra. A special small target thermocouple is provided to minimize noise, Fig. 10.

e. Mechanization

Improvements in instrumentation greatly expand the possible applications. Repeated human actions are replaced by more patient and typically more accurate mechanisms. Thus automatic recording, replacing the tedious "point by point" observations, has made possible the recent collecting of great numbers of spectra. This process of mechanization continues, freeing the human for non-repetitive actions, or making possible measurements for which the human senses are too coarse. Thus Agnew *et al.* (1951) used ten Golay pneumatic detectors at ten exit slit positions, with outputs recorded on an oscilloscope by means of a ten channel electronic switch, to follow changes in time at these wave lengths of radiations from an explosion lasting but milliseconds. The Perkin-Elmer Corporation Model 14 Infrared Automatic Multicomponent Analyzer in a 6-minute cycle measures a sample at 6 wave number positions and records the measurements on a multipoint recorder; it can be used to automatically regulate a liquid or gas chemical process stream. In utilizing infrared spectra it is not the instrument but the interpretation of the spectra which has become the major "bottleneck." This interpretation often involves the transcription of the spectrum into a series of numbers, the wave numbers of the absorption bands and their absorptivities. This might be done automatically, so that the instrument output would be not only the pen trace spectrum but also, for example, a punched IBM card.* Werner (1953) discusses one aspect of this. The "Anrad" Automatic Digital Recorder for Analog Data (Arthur D. Little, Inc., 30 Memorial Drive, Cambridge, Mass.) automatically converts analog voltages to decimal digits written on a typewriter or punched into a tape for feeding directly into computing (or memory) equipment. The "Teleducer" (Telecomputing Corp., Burbank, California) has a similar function. The "Encorder" (G. M. Giannini Co., Inc., 117E Colorado St., P.O. Box N, Pasadena, California) converts angular shaft positions into decimal digital form or signals to a Cleary printer or IBM puncher. These digital transducers would be activated at the absorption band positions, perhaps by some sort of totalling, rate of change, or matched absorbance rise and fall circuits, such as the "maximum picker" of Harrison and Molnar (1940). Once the spectral data is in digital form, it is compared to previous standard data. This too can be done automatically (see Section III). The instrument of the (near?) future will, I expect, be supplied with a built in "memory," to provide not only a spectrum but also its automatic analysis. It will have large screen oscilloscope presentation for preliminary studies and for the observation of any of the standard spectra in its memory in any quantitative combinations (see Section II 5f). Let us utilize the advances of the age that we live in.

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* I find that experiments with a similar idea were described by G. W. King and Elaine Blanton at the 1952 Ohio State Molecular Structure Symposium.

III. The Interpretation of Data

1. Absorption Theory

For a general discussion of absorption theory, see Chapter 3, by Hiskey, in this book. The major text on the theoretical utilizations of "molecular" spectra is Herzberg's (1945). The quantum energies of infrared radiations are of the order of magnitude of the energies of vibration of the atoms of a molecule. The classical picture was introduced by Bjerrum in 1916, in which the atoms were represented as point masses bound together by forces, F, which obey Hooke's law, $F = - kq$, in which k is a force constant and q is a displacement from an equilibrium position. In this first approximation the atoms are considered as harmonic oscillators, moving in simple harmonic motions at frequencies determined by their masses and by the strengths of the binding forces. Actual models, made with steel balls and spiral springs, have been useful in demonstrating the various frequencies and directions of motion which such a system can have (Kettering *et al.* 1930). If a molecule has n atoms, here pictured as mass points, its configuration in space may be represented by $3n$ space coordinates at any instant of time. Three of these may be used to represent the position of the center of mass of the molecule, and three more to represent the rotational position of the molecular axes, so that the configuration may be represented by $3n$-6 "internal" coordinates with respect to a fixed center of mass and a fixed rotation position. The vibrations may also be represented in terms of internal coordinates. If the atoms move harmonically without displacing the molecule center of mass or its rotation position, they must move in a precise manner, all with the same frequency and with definite relative amplitudes. If one atom moves to the left, another must move to the right, or else the center of mass is displaced. If one atom moves up another must move down, and lighter atoms must move farther, or more of them must move, than heavy atoms. To describe internal motions one does not need $3n$-6 coordinates for each of the many positions of the atoms, for these positions are not independent of each other; it is adequate to represent the motion of all n atoms by the frequency of the particular motion described, and an amplitude term. The possible internal motions of the system are adequately represented by $3n$-6 "normal" or "fundamental" frequencies or modes of vibration, and their amplitudes. (This is $3n$-5 for a linear molecule.) For certain highly symmetrical molecules two vibrations, involving motions of the atoms in different directions, may have the same frequency; this frequency is said to be "doubly degenerate."

A method of determining these normal modes is to move one atom then see where the other atoms would have to move in order not to displace the center of mass or rotate the molecule. Frequently the required motions are approximately along or perpendicular to the chemical bonds. It may be useful to represent the motions in terms of "displacement" coordinates, with respect to the equilibrium positions of the atoms, or by "normal" coordinates which are oriented along the directions of the normal vibrations and with scales determined by the relative amplitudes of the different atoms during the normal vibrations. The symmetry properties of the molecule may be very useful in determining these normal modes (see Herzberg, 1945). To make these assignments requires considerable training; I wish only to make the point here that each vibrational frequency involves motions at this frequency of all the atoms, although some atoms may move farther than others. However, the total energy of a vibration may be distributed more to certain atoms, with large displacements against strong restoring forces, than to other atoms, with small displacements against weak restoring forces. For example Chabbal and Barchewitz (1951) have calculated that for the C—H stretching mode of chloroform, CH_3Cl, motions of the hydrogen atoms with respect to the carbon atom account for about one million times the energy of that involved in the motion of the chlorine atom with respect to the carbon atom. On this basis, this vibration can be attributed to the C—H group, neglecting the effect of the rest of the molecule. Other chemical "group vibrations," discussed below, are not as satisfactorily assigned with a neglect of the motions of the rest of the molecule.

In terms of the wave theory of radiation, the oscillating electrical field of the radiation produces an electrical vibration in the molecule at the same frequency as that of the radiation. This molecule electrical vibration may be represented as a changing charge distribution or dipole moment. Since the forces binding electrons to atoms are greater than the forces binding the atoms together and the forces available in infrared radiations are commensurate with the latter, the primary effect of infrared absorption is to set the atoms into vibration, so that the varying molecule dipole moment produced by the absorption of the infrared radiation is primarily a periodically varying separation of the charged centers. For radiation frequencies corresponding to the normal modes of vibration, the resulting molecular vibrations are large; at other frequencies the vibrations are negligible (except at overtone and combination frequencies, to be discussed below). It may be that for symmetrical molecules some of the normal vibrations involve such a symmetrical motion of the charged centers or nuclei that there is no net change in dipole moment. Infrared radiations cannot activate these vibrations, which are spoken of as being "forbidden." For example, in ethylene, $H_2C{=}CH_2$, the normal modes of vibration which "stretch" the C=C linkage produce no change in the molecule dipole moment, and the "C=C vibration" is not observed in the absorption spectrum, although it is observed, at 1647 cm^{-1} (Randall *et al.*, 1949), in the absorption spectrum of propylene, $CH_3CH{=}CH_2$, whose lower symmetry accounts for a charge dissymmetry and hence a varying dipole moment during the vibration. Infrared absorption occurs only when it produces a periodic change in the molecule dipole moment.

The classical picture of molecular vibrations in terms of motions of a set of weights on springs explains the appearance of characteristic frequencies, or normal vibrational modes, in the infrared absorption spectrum, but inadequately explains intensities. A set of weights on springs can absorb a small or large amount of energy at a given frequency by the resulting small or large displacements of the weights. There is something about the universe which we don't yet fully understand whereby it appears that at the molecular or atomic level, energy changes are generally not continuous but involve discrete "quantized energy transitions," this interpretation being necessary to explain the observed molecular or atomic phenomena. The system changes from one "energy level" to another by the absorption or emission of a discrete "quantum" of electromagnetic energy, whose energy content, E, is given by $E = h\nu = h\,c\sigma$, in which h is Planck's constant 6.624×10^{-27} erg sec, ν is the frequency of the radiation absorbed or emitted, σ is the wave number of this radiation, and c is the speed of propagation of this radiation, 2.99776×10^{10} cm/sec in a vacuum. In this quantized world, a molecule either absorbs the full quantum energy to make the transition to the higher energy level or it absorbs no energy at all. A molecule then cannot vibrate with continuously increasing amplitudes, but for a normal vibrational energy each atom has a particular amplitude, frequency, and direction of motion.

From theoretical considerations, the energy of a molecule harmonic vibration may be represented by

$$E_{\text{vib.}i} = (v_i + \tfrac{1}{2})\, h\nu_i\,,$$

in which v_i is the "vibrational quantum number," which may have one of the integral value 0, 1, 2, 3 . . ., h is Planck's constant, and ν_i is the frequency of the i^{th} normal mode of vibration. Hence the total vibrational energy of a molecule of n atoms is

$$E_{\text{vib.}} = \sum_{i=1}^{3n-6} (v_i + \tfrac{1}{2})h\nu_i\,,$$

obtained by adding the energies of all the $3n$-6 normal vibrations. Notice that some energy remains when all the v's are zero (the "ground states"), as at a very low temperature; this is called the "zero point" vibrational energy of the molecule. At a particular temperature, the vibrational quantum numbers may have different values for the different normal modes; the probability that a particular vibration is "excited" when the system is in thermal equilibrium is given by the Boltzmann factor, $e^{-(E_{\text{vib}.i}/kT)}$, in which k is Boltzmann's constant, 1.38047×10^{-16} ergs/degree, and T is the absolute temperature in degrees Kelvin. Hence at room temperature the v's all tend to be near zero. When energy at a fundamental frequency is absorbed or emitted, the molecule changes from one discrete energy level to another, and most probably with a change of only one of the v's, of $+1$ or -1 (the "selection rules"). Hence the change in energy of the molecule is most probably equal to $h\nu_i$, and the most intense of the observed absorption bands represent the normal vibrations.

In the initial classical picture, the motions of all the atoms were considered to be harmonic, with all forces obeying Hooke's law, $F = -kq$, in which k is the force constant and q is a displacement from an equilibrium position. These forces may be represented in terms of negative derivatives of a "potential energy field," or of "potential curves." The potential energy of a molecule, or the restoring force, when an atom is considered as being drawn away from the rest of a molecule, does not increase indefinitely, but reaches a maximum value when the atom is effectively removed and the structure is ionized. A more correct potential energy versus displacement curve was mathematically described by Morse; for a discussion of Morse curves see Dr. Hiskey's chapter in this book. Hooke's law forces are no longer obeyed for large displacements, and so the vibrations become "anharmonic." Moreover, when one atom vibrates, it modifies the potential field in the region of another atom, and so the different vibrations interact, which would not be the case if the different vibrations were perfectly harmonic. The result of this is to modify the "selection rules": the vibrational quantum numbers v may change by more than $+1$, producing "overtone" absorption bands, and the v's of more than one fundamental mode may change at a time, producing "combination" absorption bands, at frequencies of $\nu_i \pm \nu_j$ in which ν_i and ν_j represent different fundamental frequencies. Because the atom motions are anharmonic, radiations at these overtone or combination frequencies can now interact with the molecule, setting it into vibration. These absorption bands are of weaker intensities than the absorption bands at the fundamental frequencies, but their presence complicates the assignment of the fundamental modes from the observed absorption spectrum.

The frequency of an overtone band is given by

$$\nu_{0v} = \nu_{00}\, v[1 - x(v + 1)],$$

in which ν_{00} represents the frequency of the fundamental mode, v is the change in vibrational quantum number for the transition from the ground state to the excited state, and x is an "anharmonicity constant," which is of the order of 0.02 for organic bonds. Mecke (1950) notably utilizes these anharmonicity constants in connection with integrated overtone band intensities to calculate bond dipole moments. These overtone bands may be of great use in analytical work, particularly with respect to hydrogen vibrations of the various types, degree of chain branching of paraffins in terms of relative numbers of CH_2 and CH_3 groups as determined from the overtone absorption bands, etc. See the review by Lauer and Rosenbaum (1952).

Table IV indicates the orders of magnitude of properties of a bond vibration, calculated in terms of the harmonic motion approximation. Carbon-carbon "stretching" vibrations, of the organic molecule "skeleton" or "backbone," are observed in the range 800–1200 cm^{-1}. For this calculation a band at 1000 cm^{-1} is considered, and it is assumed that all the energy of the absorbed quantum of this wave number is converted into vibrational energy of these two carbon atoms. For a simple harmonic motion of two

TABLE IV

A Carbon-Carbon Vibration

Approximate values

Mean bond length, 1.54 A
Absorbed quantum wave number = vibrational wave number $\sigma = 1000\ cm^{-1}$
Zero point energy ($v = 0$) of this fundamental mode, $E_0 = \frac{1}{2} hc\,\sigma = 1.0 \times 10^{-13}$ ergs
Carbon atoms mass $m = 12.0 \times 1.6599 \times 10^{-24} = 1.99 \times 10^{-23}$ grams
Bond reduced mass, in atomic units, $m' = (\frac{1}{12} + \frac{1}{12})^{-1} = 6.0$
Bond stretching force constant $k = (\sigma/1303)^2\, m' \times 10^5 = 3.5 \times 10^5$ dynes/cm
Assumed charge dissymmetry C—C^- 0.02 electrons, or 0.0961×10^{-10} esu
Mean bond dipole moment (1.54×10^{-8} cm) (0.0961×10^{-10} esu) = 0.148 debyes
Zero point vibrational bond lengths ($v = 0$) 1.46_5 to 1.61_5 A
Zero point bond dipole moments 0.141 to 0.155 D
Calculated vibrational wave number (a check of the calculation of k) $\sigma = 1303 \sqrt{3.5/6.0} = 1000\ cm^{-1}$
Vibrational frequency $\nu = c\sigma = 3.0 \times 10^{13}$ cycles/sec
Vibrational period $\tau = 1/\nu = 3.3 \times 10^{-14}$ sec
Vibrational energy ($v = 1$) $E_1 = (v + \frac{1}{2})\, hc\sigma = 2.98 \times 10^{-13}$ ergs
Maximum relative speed of motion ($v = 1$) $2V = 2\sqrt{E_1/m} = 3.5 \times 10^5$ cm/sec
Vibrational bond length ($v = 1$) 1.41 to 1.67 A
Vibrational bond dipole moments 0.136 to 0.160 D
Intensity (absorptivity) of the absorption band: weak (or variable, depending on the charge dissymmetry produced by the rest of the molecule)

mass points, the vibrational wave number σ is given by

$$\sigma = \frac{1}{2\pi c}\sqrt{\frac{k}{m'}}$$

in which c is the velocity of the absorbed radiation, 3.0×10^{10} cm/sec, k is the force constant, and m' is the reduced mass of the vibrating system:

$$1/m' = 1/m_1 + 1/m_2$$

If m′ is given in atomic weight units, then

$$\sigma = 1303\sqrt{\frac{k \times 10^{-5}}{m'}},$$

from which a force constant may be calculated, using the known masses of the carbon atoms and the observed wave number. In this manner k is calculated to be 3.5×10^5 dynes/cm, a value considerably lower than the 4.5×10^5 dynes/cm obtained from a more detailed consideration in which the motions of the atoms of the rest of the molecule (ethane) are considered. Notice that if m' were increased to 7.7 this correct k would be obtained; the carbon atoms cannot be considered as vibrating independently from the rest of the molecule. If the force constants of Table V are calculated in a similar manner from the reduced masses of only the atoms of the bond considered it will be seen that the recorded values are obtained for terminal groups or for bonds involving stronger forces than surrounding bonds, but that values lower than those recorded are obtained for internal bonds whose forces are similar to those of the surrounding bonds.

To calculate the maximum relative speed of the atoms in terms of the harmonic motion approximation, the energy E_v of the vibration is set equal to the sum of the kinetic energies of the two carbon atoms as they move through the equilibrium positions at maximum speeds, V, either toward or away from each other.

$$E_v = (v + \tfrac{1}{2})\, hc\sigma = \tfrac{1}{2}mV^2 + \tfrac{1}{2}mV^2.$$

The maximum relative speed is then $2V$. The maximum speed, V, of one of the carbon atoms in harmonic motion is also given by

$$V = 2\pi\Delta r/\tau,$$

in which Δr represents the amplitude of motion of one of the carbon atoms and τ is the period of the harmonic motion, and from this equation Δr may be calculated. Notice that the total change in separation between the two carbon atoms throughout one cycle is given by $4\Delta r$. For a simple harmonic motion, the rms (root mean squared) amplitude is given by $\sqrt{2}\,\Delta r/2$. These atomic motions occur even at room temperature or below (the "zero point" energy) requiring a modification of the scattering factors used in predicting electron or x-ray diffraction patterns. As examples, Debye (1941) and Born (1943) report on early work and Karle and Karle (1950) and Higgs (1953) report on more recent studies which take into account these vibrations. It is interesting to a biologist that Jehle (1950) suggests that these vibrations may provide the hydrodynamic forces described by Bjerknes which might account for the specificity of pairing of homologous chromosomes during germ cell formation (See the review by Schrader, 1953).

The intensity of an infrared absorption depends on the magnitude of the produced periodic change in molecule dipole moment. As diagrammatically written, a carbon-carbon bond appears homopolar, but except for highly symmetrical molecules, there may be a charge dissymmetry across this bond. For this example, it is assumed that this dissymmetry amounts to 0.02 electrons; the actual value would depend on the particular molecular structure considered. The bond dipole moment is then given by this charge dissymmetry, expressed in electrostatic units, or esu (one electron has a charge of 4.8025×10^{-10} esu), times the charge separation, or bond length, expressed in centimeters; the resultant unit of 10^{-18} esu is called the debye or *D* unit. To first approximation as used here it is assumed that this charge does not change during a vibration, and hence that the periodic change in bond dipole moment is proportional to the periodic change in bond length during the vibration. However, quantitative work requires a consideration of the variation of charge during a vibration, and inductive effects due to the bond's own field must also be considered (see Mecke, 1950). Furthermore, the intensity of the absorption depends on the change of dipole moment of the molecule as a whole rather than on the change due to one bond. For polar groups, with high charge dissymmetry, it may be adequate to add to the bond length a correction term to account for the net molecule dipole moment (Mecke, 1950), in a manner analogous to the addition of a correction term to the reduced mass of a bond structure to take into account motions of the rest of a molecule when calculating a bond force constant. For nonpolar groups or bonds, the molecule dipole moment depends primarily on the particular structure of the rest of the molecule, so that the intensities of bands attributed in approximation to the vibrations of nonpolar and usually internal groups or bonds may be quite different in different types of molecules. Notice also that a molecule need not have a permanent dipole moment in order to absorb infrared radiations, so long as the resulting vibration produces a change in dipole moment. For example, benzene has a permanent dipole moment of 0, but unsymmetrical vibrations, either in the plane or perpendicular to the plane of the molecule, produce a charge dissymmetry, and hence these absorption bands are not "forbidden," and the infrared absorption spectrum of benzene

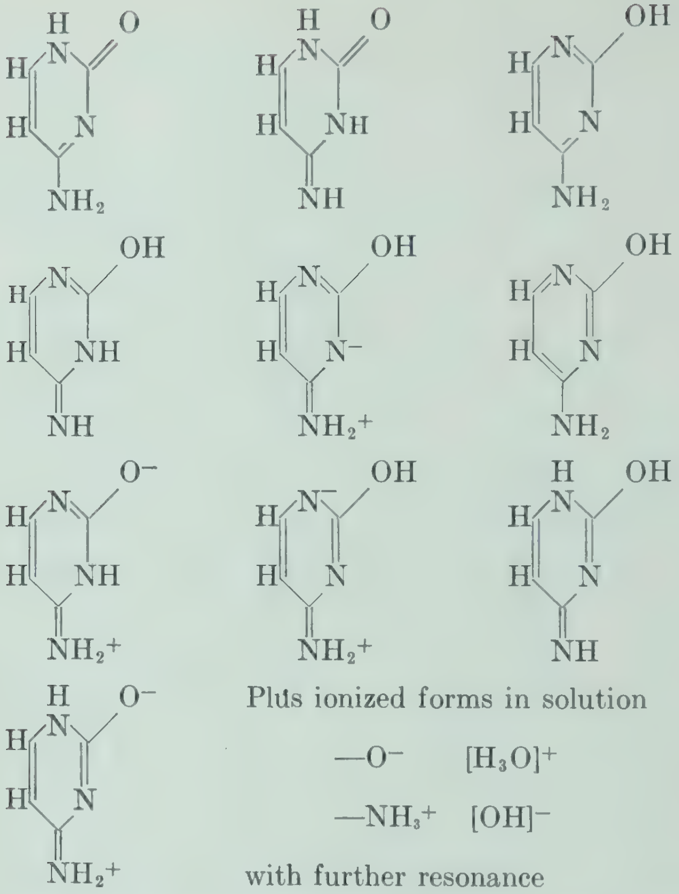

FIG. 11. Resonance and tautomeric forms of cytosine. (From Clark, 1950a.)

is quite complex. However, polar substances in general have more intense absorptions than nonpolar substances, and hence must be studied in thinner cells.

The usual diagram of a molecular structure gives little indication of charge dissymmetries which are so important in determining the intensities of infrared absorption bands as well as many other molecular properties. One method of indicating these charges is to draw a family of diagrams of a structure, as illustrated by Fig. 11. Certain of these diagrams have differing electron distributions, and likewise differing energy contents. The actual structure is pictured, notably from the work of Pauling, reviewed in 1945, as "resonating" among the various seemingly possible structures such that the properties of the structure are intermediate among those of the resonance extremes. In the quantum mechanics formulation of the electronic structure, there are terms which may be compared to the resonance structure extremes, but details of "electron" motion are not considered, and may have no significance. In a particular molecular environment, the contributions of the various resonance structures must be evaluated. Quantitative work in this field of "quantum chemistry" requires considerable training (see Eyring *et al.*, 1944), but it is recommended that a student practice writing families of structural diagrams of molecules, for these diagrams may give qualitative impressions as to the possibilities for charge dissymmetries, for bond lengths intermediate between double and single bonds, for intermediate bond energies, etc. The literalness of the resonance picture has been questioned (Burawoy, 1947; see also Coulson, 1952a), but the qualitative point of view is very helpful in relating structure to infrared spectra.

Notice that some of the diagrams of figure 11 show different positions for the hydrogen atoms, due to "tautomerism" or "dynamic isomerism." These periodic changes of hydrogen position occur in less time than most experiments (see Pauling, 1945) although with a longer period than that required for an infrared absorption process; the observed molecular properties include those of each of the tautomeric forms, with the relative importance depending on the particular molecular environment. Hunter (1945) discusses the relation of this tautomerism to hydrogen bonding. As an example, the infrared absorption spectrum of cytosine indicates both O—H and C═O vibrations, evidencing tautomerism, as qualitatively diagrammed in Fig. 11.

Quantitative infrared studies have primarily utilized measurements at the peaks of the absorption bands. From theoretical studies (see Thorndike *et al.*, 1947) it is the area under the absorption band rather than the peak height which is related to the physical phenomenon of changing molecule dipole moments as a result of the absorption process. For a sample which obeys the absorption laws, both the peak absorbency A and the area under the absorption band (plotted on an absorbency ordinate) are proportional to the concentration and thickness of the sample. However when the equivalent slit "width at half energy" of the monochromator output is as much as one half the "width at half height" of the absorption band, which not infrequently is the case in infrared measurements, Jones *et al.* (1952) have shown that the apparent peak absorptivity may be as much as 20% less than the "true" absorptivity, as measured with much smaller slit widths, whereas the apparent integrated absorptivity is only 2 or 3% less than the true integrated absorptivity, because the effect of the wide slits is not only to lower the band peak but also to increase the apparent width of the absorption band. Since the integrated absorptivity is less affected by instrument slit widths than the peak absorptivity, it is expected that integrated absorptivity measurements will have increasing use in quantitative analytical procedures, reducing the necessity of detailed calibration for each different instrument or slit width utilized. In addition, the integrated absorptivity and the band wave number give a more reliable characterization of the functional group than the wave number alone, or even the wave number and the peak absorptivity, as illustrated by Mecke (1950) for various types of C—H vibrations, as measured at the third overtone band, and by Jones *et al.* (1952) for C═O groups at various positions on the steroid nucleus.

For determining the "integrated intensity," $\int a_\sigma \, d\sigma$, which seems more properly called the integrated absorptivity, Mecke and coworkers directly planimeter the experimental curves. Richards and Burton (1949) fit the experimental curve to a modified error curve of the form $Ae^{-Bx^2-Dx^4}$, in which A, B, and D are constants and x is the wave number separation from the band center. Ramsay (1952) gives a different equation for curve shape, and includes tables for determining the integrated absorptivity in which account is taken of the finite slit widths of the monochromator. Francis (1950) has constructed a logarithmic integrator for use with transmittancy curves. Unfortunately, different authors use different units (cm/mole, 10^4 liter/mole cm^2, even cm^2/molecule second when the integration is carried out on a frequency abscissa). If the absorptivity is given in cm^2/g units, as recommended, the integrated absorptivity would be in cm/g units.

An analytical purpose of this work is to calculate the numbers of a given functional group in an unknown molecule (Jones *et al.*, 1952). The integrated absorptivity increases approximately in proportion to the number of the given functional group per unit volume. This measure may also be used to indicate the numbers of molecules in a given structural configuration, such as hydrogen bonded versus unbonded alcohols (Francis, 1951). Note also the use by Francis (1953) of an integrated absorptivity over the broad range 12.59 to 14.67 μ as a measure of the weight fraction of paraffinic methylene groups in high molecular weight hydrocarbons. In this range the individual bands are not constant in position, but the integrated absorptivity provides an adequate basis for quantitation. The purpose of integrated intensity measurements from theoretical considerations is to calculate bond dipole moments, and hence the electronic configurations of molecules. But it is not possible to add up the absorptivities of all the functional groups and so predict the intensities and wave numbers of the infrared absorption spectrum of a given molecule; many of the absorption bands are due to "the molecule as a whole," and, for molecules with perhaps twelve or more atoms or with low symmetry, cannot be predicted by present techniques.

There is a basic schism in the interpretation of infrared spectra. The theoretically minded quantum chemist or physicist develops his equations describing molecular energies from initially simple models. He emphasizes changes in the "molecule as a whole," and is unsatisfied with any neglecting of cross products or interaction terms. For complex molecules his mathematics soon swamps even a room-size electronic calculator. The empirical analyst on the other hand is satisfied to neglect theory as long as his empirical methods are useful. He treats vibrational spectra as if each band were determined by a vibrating part, or chemical group, of the molecule, each vibrating with dependence on the rest only in so far as that particular part is affected. For example he will attribute a band at 1750 cm^{-1} to a stretching vibration of a C═O group. Another band at 1730 cm^{-1} he will also attribute to C═O with the qualification that changes in other parts of the molecule have now caused less energy to be in the C═O vibration, so that the C═O bond distance would be increased and the force constant decreased. From a theoretical point of view it appears that this can only be partially true; part of this change of vibrational wave number should also be attributed to vibrational changes in other parts of the molecule. For complex molecules, the empiricist at present has the more useful picture, but quantitative structure interpretations should not neglect the "molecule as a whole."

The following examples may indicate the advantages of the joint consideration of intensities and the wave number positions of absorption bands. Normal propanol in 0.00430 M solutions in CCl_4 gives a moderately sharp absorption band at 3640 cm^{-1} with an integrated intensity of 2.28×10^{-7} cm^2/molecule sec, which is attributed to the "free" —O—H vibration (Francis, 1951). As the concentration of propanol is increased, a new broad band appears, centering at 3320 cm^{-1} but extending from 3100 to 3600, and with an integrated absorptivity of 28.9×10^{-7} cm^2/molecule sec, which is attributed to the hydrogen bonded O—H vibration. If one draws structural diagrams of the hydrogen bonded molecules, one sees that the result of the bonding is to increase O—H bond length, and hence reduce the bond force constant and the energy of the vibration, so that the absorption band is shifted to a lower wave number, equivalent to the lower quantum energy of the absorbed radiation. The broadness of the bonded O—H band indicates the varieties of possible bonding configurations and relative molecule orientations, each with slightly different energy, and this is also indicated by the diagrams. Finally the diagrams of the bonded molecules indicate increased partial charges on the bonded hydroxyl groups, so that the molecule dipole moments would be more changed by their vibrations than when the hydroxyl groups are unbonded, and the greatly increased intensity of the bonded O—H group qualitatively confirms this increase in bond dipole moment. A steroid molecule with a C═O group at the 17 position gives an absorption band at 1742 cm^{-1}, with an integrated intensity of 2.69×10^4 liter/mole cm^2. When this molecule is unsaturated between the 15 and 16 positions, conjugate to the carbonyl unsaturation, the band shifts to 1716 cm^{-1} and the intensity increases to 3.67×10^4 liter/mole cm^2 (Jones *et al.*, 1952). More of the resonance structures of the second molecule include the C—O⁻ group, with an increased bond length and hence a decreased vibrational frequency, and with an increased bond dipole moment and hence an increased intensity, than has the C═O group. A steroid molecule with a C═O group on the 3 position has an absorption band at 1717 cm^{-1}, with an integrated intensity of 2.55×10^4 liter/mole cm^2 (Jones *et al.*, 1952). On bromination at the 2 or 4 position, the band shifts to 1735 cm^{-1} and the integrated intensity decreases to 1.89×10^4 liter/mole cm^2. The resonance structures of the second molecule include a group of the form $Br^- \; C—C{\equiv}O^+$, which would have a decreased C—O bond length and hence an increased vibrational frequency. Since the properties of the structure are intermediate among those of the resonance extremes, the positive charge on the oxygen atom would partially compensate the previous tendency for a negative charge on the oxygen atom, and the change of dipole moment during the vibration would be decreased, decreasing the band intensity.

The goal of theoretical studies of molecules is to be able to describe the structure and energies of a molecule in sufficient detail to be able to predict all its properties, having established such descriptions from a minimum of previously measured properties. A description of the vibrational states of a molecule requires a knowledge of the potential field or forces at each atom, for the frequencies of vibration are determined by the magnitudes of these restoring forces. If it is assumed, as is done in the harmonic approximation, that the forces on an atom are determined only by the displacement of this atom and all others, neglecting the "cross product" effects of the displacement of one atom on the forces due to the displacement of another, then there are $(\frac{1}{2})(3n\text{-}6)(3n\text{-}5)$ force constants for a non-linear, unsymmetrical molecule of n atoms; symmetry reduces this number (see Herzberg, 1945). But there are only $3n$-6 fundamental vibrations of this molecule which are determined by these forces, the masses of the atoms being adequately known, and hence only $3n$-6 equations from which these unknown force constants must be determined. And experimentally all of these fundamental vibration frequencies may not be observed with certainty. It is necessary therefore to make additional assumptions, as to the relative importance of the interatomic forces in order to reduce the number of variables. The most widely used point of view is the "valence force field model," in which the primary forces are recognized as those which "stretch" bonds between atoms which are written in the chemical formula as being chemically bonded, but additional weaker forces are considered which restore "distortions" of bond angles, these forces being affected by interactions between different bond distortions. It is emphasized with Crawford and Mann (1950) that "force constants have meaning only in terms of the model for which they are derived"; we may expect improvements in our descriptions of molecules as our analytical and theoretical techniques allow a more inclusive comparison to related molecules. One procedure is to use the force constants determined for one molecule in the analysis of a closely related molecule, particularly one with a different atomic isotope, to allow the consideration of larger molecules or of second order effects and "cross product" terms. By such procedures, theoretical studies of the force constants of symmetrical molecules of 12 or even 18 atoms have been made (see Herzberg, 1945; Lecomte, 1949). Given the detailed force constants of a molecular structure, we could predict its vibrational frequencies, but for most molecules we can at best predict limited wave number ranges within which certain vibrational bands are expected, Fig. 12, and qualitatively predict small shifts of position or intensity of these bands with small changes of structure.

2. Chemical Group Analysis and Molecular Structure

Table V gives approximate values for some properties of the major bonds of organic molecules. The primary association should be of values on one line. Thus if two bonded atoms are in a molecular environment which increases the bond length, as suggested by greater possibilities for resonance forms with the greater bond length, one expects to find a decreased vibrational wave number, a decreased force constant, and a decreased bond energy as concomitants of this decreased association of the two atoms. Comparisons of values on successive lines cannot be made as reliably, particularly if successive bonds are of different types. Even so, for these few bonds the generalization relating bond lengths inversely to bond energies, force constants, and vibrational wave numbers does indicate major trends of the table.

As to bond energies, one has again this problem of attributing properties derived from measurements on the molecule as a whole, here the heat of atomization, to the mentally separated components. The bond energies listed in Table V are not in general those of the particular molecules listed, perhaps explaining some of the apparent anomalies of sequence. The qualitative point of view is that for a bond of a particular type a decreased bond energy, approximately that required to completely separate the atoms of a bond, is associated with a decreased vibrational energy, or vibrational wave number, required to periodically compress and partially separate the atoms of the bond. The partially filled column of the longest wave lengths of radiation which can cause bond dissociation provides another correlation.

Literature values for force constants, determined from theoretical as well as experimental considerations, are presented. These may be compared to force constants calculated from the simple relation

TABLE V

Properties of Chemical Bonds. Approximate Values

Bond	Molecule	Bond length, A	Absorption band		Force constant 10^5 dynes/cm	Group reduced mass atomic units (0 = 16.000)	Bond energy[a] k.cal./mole	Maximum dissociating wavelength[b] microns	Bond dipole moment[a] debyes
			kaysers (cm^{-1})	microns					
O—H	H_2O	0.97[a]	Stretch: 3650[c]	2.74	7.6[d]	0.95	110	(0.28)	1.51
			Bend: 1595[c]	6.27					
N—H	NH_3	1.01[a]	Stretch: 3333[c]	3.00	6.5[d]	0.94	83	(0.31)	1.42
			Bend: 1626[c]	6.15					
C—H	$CH_3CH_2CH_3$	(1.09)[a]	Stretch: 2976[c]	3.36	(5.0)[d]	0.93	(86)	(0.36)	(0.4)?
			Bend: 1469[c]	6.81	(0.54)[e]				
S—H	H_2S	1.35[a]	2688[c]	3.72	4.0[d]	0.98	82		0.67
C—O	CH_3OH	1.43[g]	1034[g]	9.67	(5.6)[g]	6.86	(75)	(0.36)	(0.7)*
C—N	CH_3NH_2	1.47[g]	1037[g]	9.64	4.86[g]	6.47	(54)	(0.40)	(0.2)*
C—C	$CH_3CH_2CH_3$	1.54[a]	870[c]	11.49	3.8[f]	6.01	(63)		
C—Cl	CH_3Cl	1.78[g]	732[g]	13.66	3.35[g]	8.97	(69)		(1.5)*
C—S	CH_3SH	1.81[g]	707[g]	14.14	3.00[g]	8.74	(54)		
C═O	$(CH_3)_2C{=}O$	(1.21)[a]	1724[c]	5.80	(12.3)[d]	6.86	(150)		(1.8)*
C═N	$(CH_3)_2{=}NOH$	(1.26)[a]	1683[c]	5.94		6.47	(84)		
C═C	$CH_3CH{=}CH_2$	(1.33)[a]	1647[c]	6.07	(9.8)[d]	6.01	(101)		
C═S	CS_2	(1.60)[a]	1522[c]	6.57	7.5[d]	8.74	107.5		
C≡N	$CH_3C{\equiv}N$	1.15[a]	2283[c]	4.38	17.5[d]	6.47	(150)		(2.5)*
C≡C	$CH_3C{\equiv}CH$	(1.20)[a]	2155[c]	4.64	(15.6)[d]	6.01	(128)		

Values in parentheses were determined from other related molecules.

* Recalculated values.

[a] Syrkin and Dyatkina, 1950.

[b] Oster and Mark, 1953.

[c] Randall *et al.*, 1949.

[d] Linnett, 1947.

[e] Crawford, and Brinkley, 1941.

[f] Bonner, 1937.

[g] Guggenheimer, 1950.

$$\sigma = 1303 \sqrt{k \times 10^{-5}/m'}\ {}^{*};$$

deviations of the calculated from the literature values indicate the inadequacy of treating the absorption as producing only a vibration of the atoms of the bond under consideration. These diviations are greatest for internal single bonds, and may exceed 25%. Fowler and Smith (1953) emphasize difficulties with C—O stretching band assignments, utilizing models to illustrate the importance of vibrations of structures as wholes. Bending force constants may be given in terms of the moment of force required to distort the structure through a given angle (Linnett, 1947, gives the C—H bending force constant as 0.5×10^{-11} dyne cm/radian) or in terms of the force required to distort the structure a given distance (C—H bending force constant about 0.5×10^{5} dynes/cm). This bending can occur in various planes, giving rise to the more specific terms of deformation, wagging, rocking, and twisting vibrations (see Randall *et al.*, 1949). In Landolt-Börnstein Vol. I, Pt. 2, 1951, Mecke and Kerkhof give symmetries, wave numbers, and in some cases force constants, bond distances, and moments of inertia for some 600 theoretically analysed molecules. In molecules other than water, the O—H bending vibrations are at lower frequencies than the 1595 cm^{-1} given in Table V, evidently contributing to two or three bands between 1000 and 1450 (see Fig. 12 and Sutherland, 1950).

The intensities of infrared absorption bands are related to the changes of molecule dipole moments during the vibrations as discussed in Section III 1. A few bond dipole moments are listed, but this table gives an inadequate representation of intensities. A perhaps suitable point of view is presented by Jones *et al.* (1952), who give an integrated absorptivity, $B = \int_{1190}^{1290\ \text{cm}^{-1}} \log\,(T_0/T)\,d\sigma$, of 3.9 to 4.7 per acetate group in steroid molecules, c being the concentration of the steroid. A perhaps more general representation of group absorptivities would have c as the concentration, in g/cm^3, of the group. Bond dipole moments vary in different molecular environments, particularly for the approximately "homopolar" bonds such as C—C, for different molecules may have quite different contributions of ionic resonance structures, i.e., quite different net charge distributions. We need to determine tables not only of frequency differences when a particular group is in different molecular environments, as the carbonyl group in esters, ketones, acids, etc., but also tables of integrated group absorptivities when the group is in different molecular environments.

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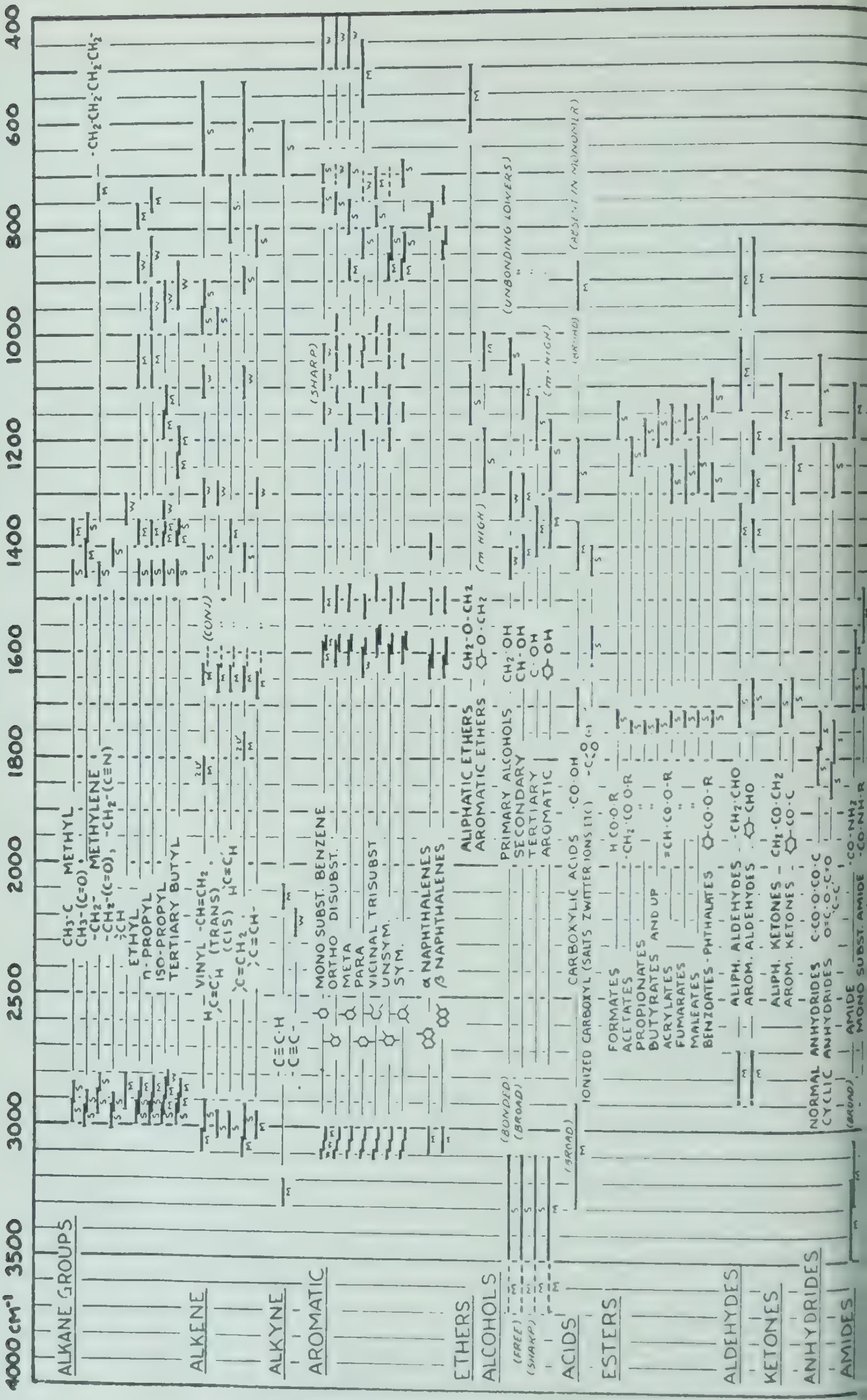
* Barnes *et al.*, (1944) give the coefficient as 1307, but evidently use an atomic weight scale based on H = 1.000. Using instead a scale based on O = 16.000 and using Binge's 1941 physical constants (see Herzberg, 1945, page 538), the coefficient is calculated as $1303._1$.

A number of more detailed correlations have been suggested for bonds of particular types. Guggenheimer (1950) empirically relates stretching force constants to a negative exponential power of the bond distances for diatomic and certain symmetrical polyatomic molecules, with a term included of the numbers of outer orbit electrons. Walsh (1947) relates carbonyl bond distance to force constant, bond energy, and polarity. Gordy (1947) relates bond order to bond distance and energy for C—O and C—C bonds. Bond order is a number representing an intermediate bond condition, usually between a single bond of bond order 1 and a double bond of bond order 2. Linnett (1947) suggests a general relation, $kR_n = C$, in which k is a force constant, R is the equilibrium bond distance, and n and C are characteristics of particular bond types.

Figure 12 is Colthup's modification of a figure in his 1950 paper. Molecules containing the listed functional groups usually have infrared absorption bands at the listed positions, as well as other bands, particularly in the region of 9 to 15 μ, due to the "molecule as a whole." Limited regions for the group absorption bands are listed; the exact position of the band within the region is affected by the structure of the rest of the molecule. It is the goal of this spectra-structure correlation work to be able to predict the exact structure of a sample from its infrared absorption spectrum, in addition to its atomic and molecular weight analyses. The procedure is to subdivide the correlations made with molecules of known structure and spectra not only with regard to the major functional group but also with regard to the local molecular environments of this functional group. For example, notice the subdivisions of the C═O stretching vibration assignments. The student is encouraged to interpret these "perturbations," or small shifts of absorption band positions due to structural changes of minor influence, in terms of resonance diagrams and other bond properties, as listed in Table V. The limit of this subdivision work is to have in the files a spectrum of a known molecule which is identical with that of the unknown; the structure then is completely known, to the extent that the molecule has been studied or described in the world's literature, without the possible ambiguities of the limited spectra-structure correlations. It is encouraged that spectroscopists contribute their efforts to the compilation of an international catalog of the infrared spectra of all known molecules, on the basis that most of us are studying molecules which have been previously studied in some regard. However, these spectra-structure correlations are of use until such inclusive catalogs are available, and particularly will remain of use in the study of newly isolated or synthesized molecules. It is suggested that the infrared spectra of all such molecules be systematically added to the international catalog. It is expected that machine correlations might be made between the spectrum of an unknown molecule and the spectra of groups of molecules of known and related but not identical structures, greatly extending the correlations illustrated by Fig. 12. Bellamy (1954) presents a more detailed review.

Figure 12 indicates that most functional groups have absorption bands in several regions of the spectrum, due to their several parts or to bending as well as stretching vibrations. This is of particular use, for a single band might be due to a great many different groups. The tabulations of empirical and theoretical band assignments in Randall *et al.* (1949) offer extensive examples, very useful for comparisons to particular spectra, of this possible ambiguity of assignment. One should examine the entire spectrum; it is unfortunate that published spectra frequently show only limited regions. Even the entire spectrum may not be adequate to give unique group identifications in terms of the detail of Fig. 12. One can usually say only what groups might be present and what groups probably are not. The systematic procedure then is to prepare derivatives utilizing predictable chemical reactions and study their spectra. For example, the contributions of C═C groups to an absorption band may be determined by studying the spectra of bromination or hydrogenation products (see Cross *et al.*, 1950). These authors also suggest that a band may be assigned to a given group if the graph is linear of the absorptivities at the band position versus the proportional numbers of this group in molecules of known structure and of similar type. Kitson (1953) suggests forming cyano as well as halo derivatives of C═C groups. Vibrations of groups which are ionizable may be studied by modifying the degree of ionization, in aqueous acid or alkali. In morpholine, the carboxyl group may be ionized. One may also make methyl esters or salts of acids. Hydrochlorides may be formed from amines, modifying and hence identifying N—H vibrations. Hydroxyl groups may be acetylated. These large addition groups commonly affect the properties not only of the local molecular region of reaction. Other bands may be displaced as well as those due principally to the initial reactive group. Conclusions are most safely ones of exclusion: if a band is not affected in a major way when a reaction is carried out which greatly modifies a particular group, then the band is not due to that group. Substitutions which involve less drastic modification of the parent compound involve isotope replacement, which presumably but slightly modify force constants yet modify mass, and hence vibrational wave number, in a predictable manner. Deuteration is particularly useful (see Halverson, 1947), decreasing vibrational wave numbers of bands due to hydrogen stretching or bending vibrations approximately by the factor $1/\sqrt{2}$. For "labile" hydrogen atoms, particularly those which have ionized resonance forms and so can contribute to inter or intramolecular hydrogen bonds, such deuteration may be carried out simply by putting the sample in D_2O. The mass changes for other isotope substitutions are less, requiring instruments of higher resolution, and greater care, to identify


4000 cm⁻¹
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
ALKANE GROUPS
CH₃·C
CH₃·(C=O)
METHYL
-CH₂-
-CH₂-(C=O), -CH₂-(C≡N)
METHYLENE
⩾CH
-CH₂·CH₂·CH₂·CH₂-
ETHYL
n-PROPYL
ISO-PROPYL
TERTIARY BUTYL
ALKENE
VINYL -CH=CH₂
C=C (TRANS)
(CIS)
C=CH₂
C=CH-
(CONJ)
ALKYNE
C≡C·H
C≡C-
AROMATIC
MONO SUBST. BENZENE
ORTHO DISUBST.
META
PARA
VICINAL TRISUBST
UNSYM.
SYM.
(SHARP)
α NAPHTHALENES
β NAPHTHALENES
ETHERS
ALIPHATIC ETHERS
AROMATIC ETHERS
CH₂·O·CH₂
⌬·O·CH₂
(m HIGH)
ALCOHOLS
(FREE)
(SHARP)
(BONDED)
(BROAD)
PRIMARY ALCOHOLS
SECONDARY
TERTIARY
AROMATIC
CH₂·OH
CH·OH
C·OH
⌬·OH
(m-HIGH)
(UNBONDING LOWERS)
ACIDS
(BROAD)
CARBOXYLIC ACIDS
·CO·OH
IONIZED CARBOXYL (SALTS ZWITTERIONS ETC)
-C(=O)O(-)
(ABSENT IN MONOMER)
ESTERS
FORMATES
ACETATES
PROPIONATES
BUTYRATES AND UP
ACRYLATES
FUMARATES
MALEATES
BENZOATES · PHTHALATES
H·CO·O·R
·CH₂·CO·O·R
=CH·CO·O·R
⌬·CO·O·R
ALDEHYDES
ALIPH. ALDEHYDES
AROM. ALDEHYDES
·CH₂·CHO
⌬·CHO
KETONES
ALIPH. KETONES
AROM. KETONES
CH₂·CO·CH₂
⌬·CO·C
ANHYDRIDES
NORMAL ANHYDRIDES
CYCLIC ANHYDRIDES
C·CO·O·CO·C
AMIDES
AMIDE
MONO SUBST. AMIDE
·CO·NH₂
·CO·NH·R
(BROAD)
S
M
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2V

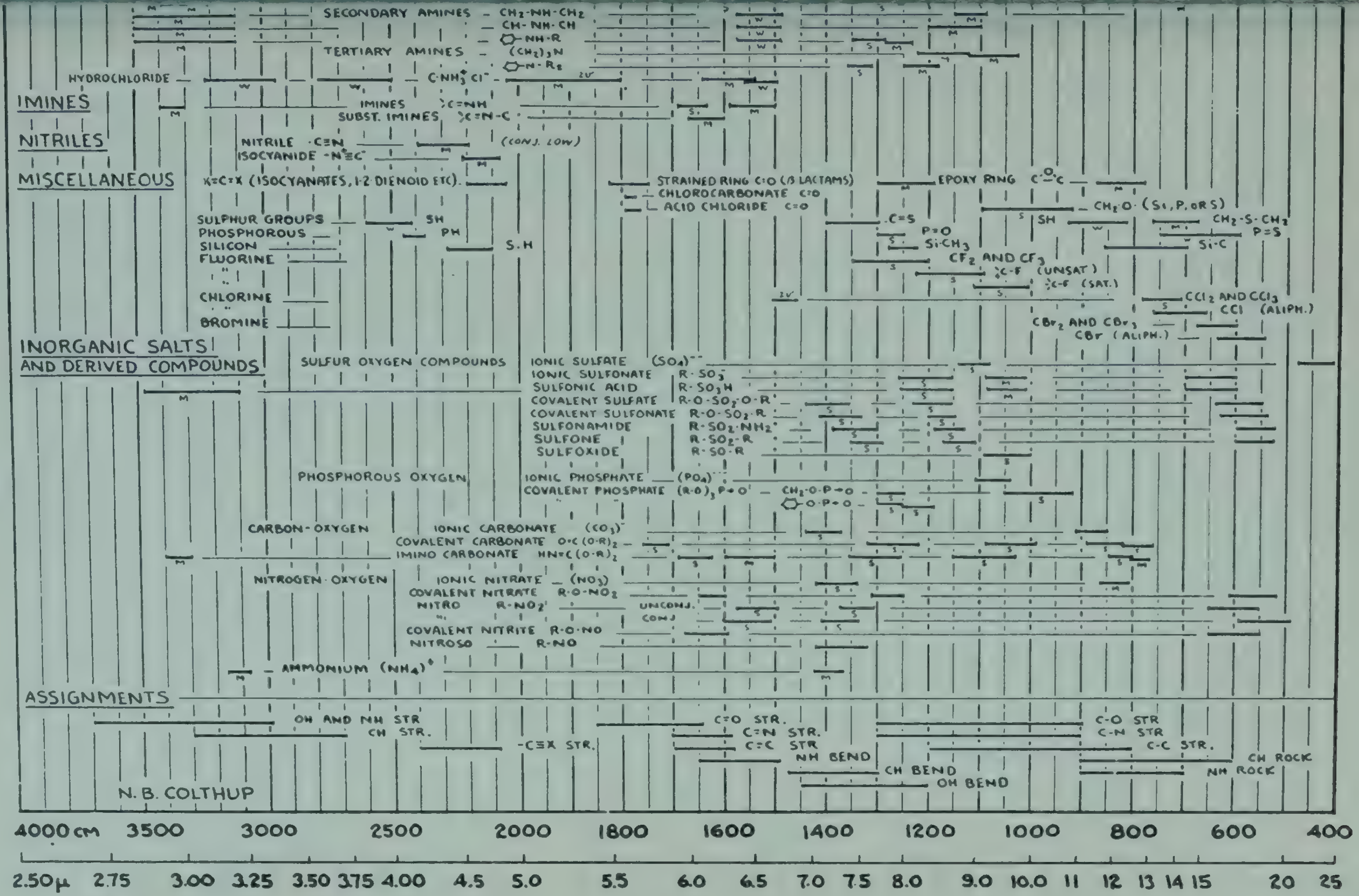


Fig. 12. Functional group analysis. (From Colthup, 1950, *J. Op. Soc. Amer.* **40**, 397, courtesy of the author, American Cyanamid Co., and the editor.)

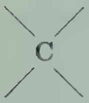
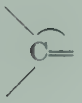
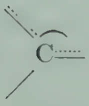
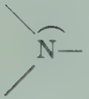
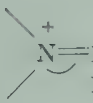
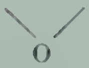
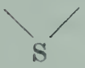
and interpret the band shifts. Finally, it is the goal of the spectroscopist to be able to predict the change in spectra for any change in molecular structure on the formation of any derivative. The "quantum" chemists are making the greatest strides toward this goal (see Coulson, 1952). The study of the spectra of derivatives of an unknown molecule is analogous to the study of the melting points of the derivatives; if one really knows the structure of a molecule, one also knows the derivatives which are formed by previously studied reactions and hence should be able to observe the properties of these previously studied derivatives.

Table VII gives the formulae of the more important biochemical groups. It is recommended that the student construct these groups, using molecular models or soldered pieces of wire, to gain insight into their three dimensional structures and possibilities for changes of structure. Approximate bond distances are given in Table V, and bond angles in Table VI. In actual molecules, different or intermediate bond distances or angles may be found, as suggested by the seemingly possible reasonance forms. Small molecules may have free rotation about single bonds in the gaseous state, but in the liquid or particularly the solid state, this free rotation in general does not occur. Different intra and intermolecular configurations have different energies and are separated by "energy barriers," or the excitation energy required before the system can change to a new configuration. "Rotational isomers," or isomers which differ only as to the rotational configuration of the various groups, may be observed (see Brown and Sheppard, 1950). It is recommended that the student visualize molecules as having definite sizes and shapes. For solids, see particularly Wyckoff (1948, 1951, 1953) and the journal *Structure Reports*. In the liquid phase this crystal structure is not perfectly maintained, although to first approximation for organic molecules the intramolecular structure is the same. Intermolecular orientations "tend" to be like those of the crystal, and for polar substances, with high intermolecular association even in the liquid state, crystallite organization may extend for several molecules.

Molecules are named in terms of a parent compound and substitution groups, one of which if a "principal function" is given in the suffix and the others in the prefix. Table VIII lists the major principal functions of biochemicals; if a molecule has more than one of these, the one highest in the list is given in the suffix and the others are given in the prefix. Table VII gives the prefix names of the major biochemical groups. It would be of value to add to this table the wave numbers of the expected absorption bands, but this information is already largely included in Fig. 12. In contemplating inclusive infrared catalogs one can see the necessity of using systematic molecular nomenclature, so that all workers can determine from a name the same and a unique molecular structure (or, ideally, the appor-

TABLE VI

Approximate Bond Angles

	109° 28′ (tetrahedral)
	124° (planar)
	120° or 108° in resonating 6 or 5 membered rings (planar)
—C≡	180°
	108° (trigonal pyramid, in NH_3) to 108° or 120° (planar, in resonating structures)
	127° (as in the nitro group, planar; to tetrahedral in NH_4^+) 120° or 108° in resonating rings (planar)
—N=	180° (to 120° or 108° in resonating rings)
	110°
	106°
$CO_3^=$	trigonal planar, C—O 1.25A*
NO_3^-	trigonal planar, N—O 1.22A*
$PO_4^≡$	tetrahedra, P—O 1.56A*
$SO_4^=$	tetrahedra, S—O 1.65A*

The last two are no longer equilateral tetrahedra when one or more oxygen atoms are covalently bound to some other atom, as in incompletely ionized and noncrystalline forms. For phosphates, reasonable values are P=O 1.39A, P—O 1.61A, O—P—O 102°, O—P—O 116° (see Gore, 1950, Discussions Faraday Soc. **9**, 138.)

* Wyckoff, Crystal Structures, 1948, 1951.

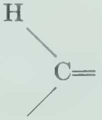
tioned weights of the resonance structures) for the conditions of the experiment. For a more complete discussion, see "The Naming and Indexing of Chemical Compounds," Chemical Abstracts **39**, 5867–5975, 1945, also available from the American Chemical Society as a reprint.

3. Spectra and Molecular Environments

In Section III 1, it was described that infrared absorptions produce vibrations of the molecular system which can be considered only in approxi-

TABLE VII

Biochemical Groups

Prefix name	Group	Prefix name	Group
acetoxy	$CH_3C(O)O-$	guanyl	$H_2NC(=NH)-$
acetyl (aceto)	$CH_3C(O)-$	hydroxy	$HO-$
(acyl)*	$RC(O)-$	imino	$HN=$
(alkane or alkyl)*	H—C saturated	keto (oxo)	$O=$
(alkene or alkylene)*		mercapto (sulfhydryl)	$HS-$
(alkyne)*	$H-C\equiv$	methoxy	CH_3O-
allyl	$CH=CHCH_2-$	methyl	H_3C-
amido, amino	H_2N-	methylene	$-CH_2-$
benzyl	$C_6H_5CH_2-$	nitro	O_2N-
carbamyl	$H_2N\ C(O)-$	nitroso	$O=N-$
carbonyl	$O=C$	oxalo*	$HO(O)C-C(O)-$
carboxy	$HO(O)C-$	phenoxy	C_6H_5O-
cyano	$N\equiv C-$	phenyl	C_6H_5-
ethoxy	C_2H_5O-	sulfiniyl	$OS=$
ethyl	C_2H_5-	sulfo	HO_3S-
ethylene	$-CH_2CH_2-$	sulfonyl	$-SO_2-$
formyl	$HC(O)-$	thio carbonyl	$SC=$
guanido	$H_2NC(=NH)NH-$	thiol (S replacing O in OH)	$HS-$
		thiono (S replacing O in CO)	$SC=$
		ureido	$NH_2C(O)NH-$
		vinyl	$H_2C=CH-$

* Not listed in Chem. Abstracts, Group Tabulation, **39**, 5963–5966, 1945.

TABLE VIII

Biochemical Groups

Principal functions, in sequence of their use in the suffix of the chemical name

Suffix name	Group
(carboxyl)ic acid or -oic acid	$HO(O)C-$
amide (replacing OH of acid)	H_2N-
al (aldehyde)	$O=$
one (ketone)	$O=$
ol (alcohol)	$HO-$
thiol	$HS-$
amine (replacing H, or attached to a non-acid radical)	H_2N-

(Part of Paragraph 68, Chem Abstracts **39**, 5876, 1945).

mation as vibrations of different parts, or chemical groups, of the molecule. If the molecular system has regular or favored intermolecular associations it is also an approximation to consider the vibrations as due only to individual molecules; one must consider the "system as a whole." It is not surprising then that as the molecular environments are varied, as by changes of state, temperature, pressure, externally applied fields, different solvents, etc., there are changes in the spectra of the molecular system.

In the gaseous state, densities are at most of the order of $\frac{1}{1000}$ of the densities of the liquid or solid, and intermolecular distances are increased by about a factor of 10. At lower pressures intermolecular distances are further increased, decreasing the frequency of intermolecular collisions and in general making negligible intermolecular associations. The molecule is now free to rotate, with different rotational energies corresponding to different rotational inertias about the three principal molecular axes. These rotational energies are quantized, so that a molecule has characteristic "pure rotation" absorption bands in the "microwave" electromagnetic region near one centimeter wave length, or a wave number of 1 cm^{-1} or a frequency of 3×10^{10} cycles per second (0.03 megamegacycles). Microwave spectroscopy utilizes primarily radio rather than optical techniques; see Gordy (1953). Molecules may simultaneously have rotational and vibrational energies, so that as different molecules change from one particular vibrational energy state to another they may change to different rotational energy states. Since the rotational energies, corresponding to 1 cm^{-1}, are smaller than the vibrational energies, corresponding to 1000 cm^{-1}, rotational energy levels may appear as "fine structure" on the vibrational absorption bands; these bands will have superimposed on them a large number of smaller closely spaced bands, with the complex being called vibrational-rotational bands. In the liquid state, molecular collisions are sufficiently frequent to prevent in general these quantized rotational states. This then is the major difference between spectra in the liquid or solid state and spectra in the gaseous state for the "same" molecule (same to the extent of the primary valence bonds): the spectrum of the vapor has many more absorption bands, as "rotational fine structure" superimposed on the vibrational absorption bands. Studies of rotational fine structure can provide important information as to the molecular size and shape (see Herzberg, 1945). The vibrational bands themselves may also be affected by the change from liquid to gas, particularly those bands which are related primarily to changes of vibration of groups affected by intermolecular association. As an extreme example, the frequencies of hydrogen-halide vibrations may change as much as 10% with change of state (Harrison, *et al.*, 1948).

Molecular associations are most intimate and constant in form in the solid state. For ionic crystals, particularly those with but two atom types such as NaCl whose "molecular" size may be considered as that of the crystal, vibrations must be considered in terms of motions of the crystal as a whole. These "lattice" vibrations, in which all equivalent atoms move in phase, have wave numbers of the order of 200 cm^{-1}, and these vibrations may be the only ones for the "infrared optics" salts (Table I), explaining their high transparency in the mid-infrared region. For molecular crystals, in which intermolecular forces can be distinguished and are appreciably less than intramolecular forces, vibrations may be attributed in first approximation a) to the molecules, and b) to the lattice, or to point groups or symmetry associations within the lattice. These vibrations are anharmonic and hence coupled, producing combination bands as well as the fundamentals (see Hornig, 1950). In general, we expect crystal spectra to be more complex than liquid or solution spectra, although here also many molecular vibrational bands are shifted by small amounts. In crystals with more than one molecule per unit cell, these different molecules may not be crystallographically equivalent, by symmetry operations, and so will have slightly different structures. These may be sufficiently different to cause a "splitting" of the vibrational bands, with the different structures having slightly different vibrational wave numbers. On the other hand, "selection rules" apply to the crystal that do not apply to the liquid (see Winston and Halford, 1949), which tend to simplify the spectra. Kletz and Price (1947) present the spectra of 12 alkylphenols in liquid and solid states. On formation of the crystals, some bands split into doublets or even triplets and some new bands appear. Bands may be shifted to higher wave number (particularly deformation vibrations) or to lower wave number (particularly valence vibrations), with shifts of the order of 5 cm^{-1}. The crystal bands are "sharper," with a reduced band width, attributed to the reduced collision broadening, but are less intense than the liquid state bands. For molecules with possible rotational isomers with approximately equal energy contents, several isomers may be present in the liquid state, each with different absorption bands, but only one in the crystal state. When the crystal state spectrum has fewer bands than the liquid state spectrum, increased isomerism in the liquid is suggested (see Brown and Sheppard, 1950).

A consideration of the infrared spectra of crystals in connection with spacing data, as determined from x-ray diffraction studies, may give further insights into molecular structure. As an example, the position of the hydrogen bonded O—H vibration in the liquid state is about 300 cm^{-1} lower than the position of the free O—H vibration, as measured in dilute solution (Francis, 1951, discussed in Section III, 1). In crystals, hydrogen bonds may be even stronger, with separations of the bonded O—H vibrations from the free O—H vibration, as measured in dilute solution, of 925 cm^{-1}

for adipic acid, with an intermolecular O—H——O distance of 2.68 A to as much as 1700 cm^{-1} for maleic acid, with an O—H——O distance of 2.47 A (Lord and Merrifield, 1953).

Temperature affects the number of molecules in a given energy state E by the relation $ge^{-E/kT}$, in which g is the "degeneracy" of the state, or number of different dynamic configurations with this energy, and k is Boltzmann's constant, 1.3805×10^{16} erg/degree. The intensity of an absorption band, related to the transitions of molecules between two energy states separated by the absorbed quantum energy, depends on the numbers of molecules in the initial state. Since in the infrared region transitions with a change of vibrational quantum number v of 0 to 1 have very closely the same wave number separation as transitions with a change of v from 1 to 2, or even 2 to 3, this effect of temperature on spectra with respect to the numbers of molecules in a given initial vibrational state is not great, in contrast to the effects on vibrational fine structure of electronic spectra, as measured in the ultraviolet region (see Harrison *et al.*, 1948). Of greater significance are the effects of temperature on the energies E of the vibrational states. Intermolecular associations are decreased at higher temperatures (usually markedly so when there are changes of state), affecting the vibrational energies of atoms or groups involved in such associations. As an extreme example, Fox and Martin (1940) report that the band of liquid water near 2130 cm^{-1}, related to intermolecular aggregations by hydrogen bonds, is shifted 54 cm^{-1} to lower wave number when the temperature is raised from 3 to 70°C. Holliday (1950) reports a shift to lower wave number of 30 cm^{-1} of the N—H band of nylon, near 3300 cm^{-1}, as the temperature is raised from 100 to 300°C, interestingly without a marked change of the curve of wave number shift versus temperature at the melting point, 255°. Other bands less involved in intermolecular associations are shifted less. Temperature also affects absorption band widths, by affecting collision frequencies in liquids and gases and by the "Doppler broadening" effect whereby slightly different wave lengths are absorbed depending on whether the molecule is moving toward or away from the incident radiation. At low temperature additional bands may be resolved due to this narrower band width. Hainer *et al.*, (1949) studied cholesterol at 80° K, and report ten bands between 798 and 844 cm^{-1}. In Fig. 13 of cholesterol in solution at room temperature, only two of these are seen, with two more as side bands. Low temperature studies of biochemicals whose room temperature spectra have inadequate detail may be very significant for their identification and structural elucidation.

Pressures up to 50 atmospheres may be useful to produce "pressure broadening" of the rotational fine structure of the spectra of gases, to obscure this fine structure and allow a more accurate integrated absorptivity

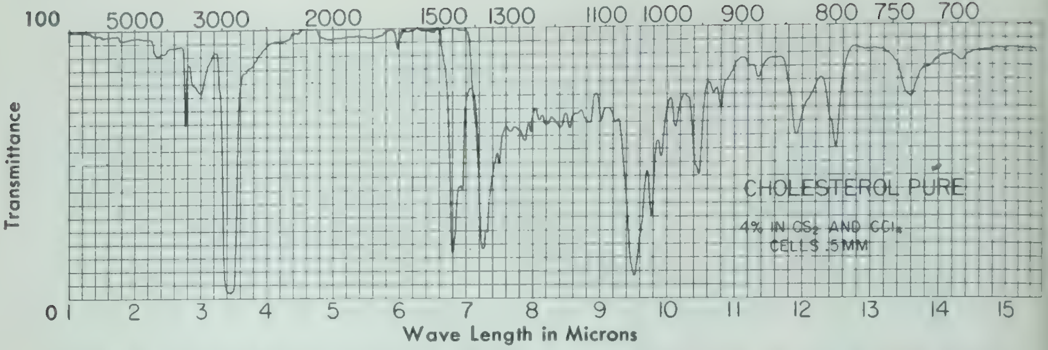


FIG. 13. The infrared spectrum of cholesterol. (Courtesy of the Perkin-Elmer Corporation)

measurement, of use in calculating bond dipole moments (Thorndike *et al.*, 1947). Pressure effects on the infrared spectra of liquids (Collins, 1930) and the effects of electrical and magnetic fields are not appreciable with present techniques.

Different solvents may modify spectra, affecting solute-solute and solute-solvent interactions, and even ionizations. The method of Ashdown and Kletz (1948) is notable: the spectra of mixtures of aldehydes and alcohols were compared to the spectra of the pure substances in separate cells in series. The formation of hemiacetals was postulated as the result of solvent-solute interaction on the basis of spectral changes. Increasing concentrations of alcohols in nonpolar solvents show increasing absorptivities of the "bonded O—H" absorption band, evidencing the increasing hydrogen bonded solute-solute interactions (Francis, 1951). Solvations and solvalytic effects, notably ionizations, also modify spectra; for example the ionized carboxyl group, $C(O)O^-$, no longer shows the O—H vibration found for the un-ionized group, C(O)OH (see Fig. 12). These modifications of chemistry with concentration or solvent make difficult the application of the absorption laws. For nonpolar substances, absorptivities may apply over large concentration ranges or even in different solvents, but for polar substances this may not be true; in reporting quantitative work the particular conditions of the measurement as well as the calculated absorptivities should be given. The solvents used in obtaining solution spectra should be designated, for the spectra with different solvents may be different.

In studying perturbations, brought about by either chemical or physical means, a distinction should be made between absorption bands which are "shifted" and new absorption bands. Shifting implies that a very similar vibrational analysis may be made, though with slightly different energies, and one expects that intermediate shifts may be found when lesser perturbations are applied. A new band requires a quite different vibrational analysis, and intermediate bands between the old and the new are not expected. This distinction is one of degree, but in empirically comparing the spectra of two molecules it may not be correct to say that an absorption band of one is shifted to a position of the other, for the association of these bands may be fortuitous and the bands related to quite different vibrational modes.

4. Polarization Spectra

Polarization errors are discussed in Section II 3g. Polarization accessories are discussed in Section II 6c. The science of infrared crystallography is rapidly developing, notably, in the United States, with the work of Hornig (see 1950) and Halford (see Winston and Halford, 1949) and students. A theoretical analysis of a structure of known dimensions and force constants gives its fundamental modes, both as to frequencies and directions of vibration. A plane polarized beam of radiation at a fundamental frequency is absorbed to greatest extent when its electric vector vibration is along the direction of net dipole moment change of the funadmental vibration. Polarized infrared spectra of crystals of theoretically analysed molecules can establish the orientations of these molecules in the crystallographic unit cell. This technique then implements x-ray diffraction techniques, and may be particularly useful for orienting bonds, especially those involving hydrogen, which are oriented with difficulty by x-ray methods. For crystals of known structure, polarization spectra may allow the assignment of the observed absorption frequencies to particular fundamental modes, or in approximation to particular group vibrations. (Mann and Thompson, 1948). For molecules which have not been theoretically analysed, it is assumed in approximation that the greatest absorption of a frequency attributed to the stretching vibration of a particular chemical group occurs when the electric vector of the radiation is along the bond of the group rather than along the unknown direction of net dipole moment change of the molecule or crystal as a whole. Analyses of the local region of a group may provide a better approximation of the orientation as determined from the dichroism of the absorption; Price and Fraser (1953) treat the carbonyl group of proteins in terms of a bent triatomic N—C=O structure, and conclude that the greatest absorption of the 1650 cm^{-1} "carbonyl" band is for an electric vector displaced 10 to 20° toward the N—C direction from the C=O bond. Note that if there is more than one molecule per crystallographic unit cell, the groups of these molecules are not all oriented in parallel. Moreover, even if there is but one molecule per unit cell but this molecule contains several like groups not oriented in parallel, the polarization spectra characterize the averaged effects of the vibrations of all the like groups, from which a unique orientation of each may not be derivable. Polarization spectra characterize the unit cell rather than the molecule, and may represent averaged effects not obtainable with the single molecule or group.

Polarization spectra are most conveniently measured with large flat crystals. When there is oblique incidence, computations must be made as to the refraction effects in determining the path lengths of the waves in the crystal, and these may be different for ordinary and extraordinary components. Moreover, the infrared refractive indices may not be known. If the crystal does not have suitable natural cleavage, the necessary surfaces may be scraped and polished after mounting the crystal in a supporting medium. The Miller (crystallographic orientation) indices of the face measured must be known to relate the polarization data to other data on structure; this work should be paralleled by optical and/or x-ray crystallographic studies on the same material. If the crystal is small, a microscope may be necessary to pass sufficient energy through it for the measurement, and a microscope is also useful or even essential in that crystals which are thin enough for study do not have to be several millimeters across, and so naturally grown rather than tediously polished crystals may be studied. As the numerical aperture of the microscope increases, the polarization data represent averages over increasingly broad angles of incidence, and the expected axial dichroic ratios are not obtained.

With plane polarized radiation incident on the crystal, the procedure is to determine the orientation of the polarizer with respect to the crystal face which gives a maximum absorption at a given frequency, then determine this absorption and the minimum absorption, determined with the polarizer 90° from this position. The "dichroic ratio" is the ratio of the absorbancies or absorptivities of these measurements. If the sample has a unique fiber or crystal axis, one may speak of parallel or perpendicular dichroism depending on whether the absorption is greater with the electric vector of the radiation parallel or perpendicular to this axis. To determine the necessary orientation of the polarizer for a sample without a morphologically obvious unique axis, an analyzer beyond the crystal would be useful, although analyzers have not been commonly used in polarized infrared studies, perhaps because of difficulties of reproducibly positioning them inside the monochromator or in front of the detector. However, Gutowsky (1951) used an analyzer to study the optical rotation of quartz in the infrared region, and reports an anomalous rotary dispersion beyond 3.7 μ as the longer wave length absorption bands are approached. The maximum and minimum absorptivities and their orientations with respect to the crystallographic axes should be determined for the other absorption bands, and for other crystal faces. The description of the variation of absorption with angle and wave length has considerable similarities to the description by means of the indicatrix of the variation of refractive index with angle and wave length.

In the wave length region of an absorption band, the refractive index does not decrease smoothly with wave length (normal dispersion) but rather goes through an abrupt fall and rise (anomalous dispersion) whose magnitude is different for different absorption bands which characterize different effects on those aspects of molecular structure which determine refractive index (see Ditchtburn, 1952). In a theoretical analysis of the interaction of radiation, described by Maxwell's equations, with matter, described by

TABLE IX

Absorptivitrix Symmetry

Crystal system	Unit cell	Absorptivitrix
Cubic	$a = b = c$ $\alpha = \beta = \gamma = \frac{\pi}{2}$	$a_\alpha = a_\beta = a_\gamma$ $\phi_{aa_\alpha} = \phi_{ba_\beta} = \phi_{ca_\gamma}$ Isotropic
Tetragonal	$a = b \neq c$ $\alpha = \beta = \gamma = \frac{\pi}{2}$	$a_\alpha = a_\beta \neq a_\gamma$ $\phi_{aa_\alpha} = \phi_{ba_\beta} =$ any angle up to $\frac{\pi}{2}$ $\phi_{ca_\gamma} = 0$ Uniaxial
Hexagonal	$a = b \neq c$ $\alpha = \beta = \frac{\pi}{2}$ $\gamma = \frac{2\pi}{3}$ (For convenience of indexing, four axes c, a_1, a_2, a_3 may be used, with a_1, a_2, a_3 perpendicular to c and each making an angle of $\frac{2\pi}{3}$ with the others. These crystals have a sixfold axis of rotation symmetry along c.)	$a_\alpha = a_\beta \neq a_\gamma$ $\phi_{aa_\alpha} - \phi_{ba_\beta} + \frac{\pi}{2} = \frac{2\pi}{3}$ $\phi_{ca_\gamma} = 0$ Uniaxial
Trigonal or Rhombohedral	$a = b \neq c$ $\alpha = \beta = \frac{\pi}{2}$ $\gamma = \frac{2\pi}{3}$ (For convenience of indexing, four axes c, a_1, a_2, a_3 may be used, with a_1, a_2, a_3 perpendicular to c and each making an angle of $\frac{2\pi}{3}$ with the others. These crystals have a threefold axis of rotation symmetry along c. Indexing is less commonly given in terms of the rhombohedral cell $a' = b' = c'$, $\alpha' = \beta' = \gamma' =$ obtuse; c is the short volume diagonal of the rhombohedral cell.)	$a_\alpha = a_\beta \neq a_\gamma$ $\phi_{aa_\alpha} - \phi_{ba_\beta} + \frac{\pi}{2} = \frac{2\pi}{3}$ $\phi_{ca_\gamma} = 0$ Uniaxial

Crystal system	Unit cell	Absorptivitrix			
Orthorhombic	$b > a \neq c$	$a_\gamma > a_\beta > a_\alpha$			
	$\alpha = \beta = \gamma = \frac{\pi}{2}$	a_γ	along c	along b	along a
(In class mm, the c axis is unique. In classes mmm and 222, $b > a > c$.)		ϕ_{ca_γ}	0	$\frac{\pi}{2}$	$\frac{\pi}{2}$
		ϕ_{ba_β}	0 or $\frac{\pi}{2}$	$\frac{\pi}{2}$	0 or $\frac{\pi}{2}$
		ϕ_{aa_α}	0 or $\frac{\pi}{2}$	0 or $\frac{\pi}{2}$	$\frac{\pi}{2}$
		(At particular wavelengths, certain ϕ's may change by $\frac{\pi}{2}$: crossed axial dispersion) Biaxial			
Monoclinic	$a \neq b \neq c$ $a > c$ $\alpha = \gamma = \frac{\pi}{2}$ β obtuse (b axis unique)	$a_\gamma > a_\beta > a_\alpha$ a_α or a_β or a_γ is along b. The other two ϕ's vary with λ. Biaxial			
Triclinic	$b > a > c$ $\alpha \neq \beta \neq \gamma$ α, β obtuse	$a_\gamma > a_\beta > a_\alpha$ All three ϕ's vary with λ. Biaxial			

For specifications of the inequalities, see Donnay, 1943, *Amer. Mineral.* **28**, 313.

electron densities and equations of motion, a complex refractive index $a = n(1 - ik)$ is required, in which n is the real part of the refractive index and k is an absorption coefficient. The optical properties of an absorbing crystal might be described in terms of a "complex indicatrix," which gives a measure of both the real part of the refractive index and the absorption, but such a figure cannot be constructed in three dimensional space. It is convenient therefore to treat the refractive index and the absorption as independent, as they almost are at absorption bands at which the anomalous dispersion effects are slight. One has then a three dimensional indicatrix, the radii of which represent the refractive indices for waves whose electric vectors vibrate along these radii, and an absorption surface, for which I suggest the name "absorptivitrix," the radii of which represent the absorptivities, in cm^2/g, for waves whose electric vectors vibrate along these radii. When the anomalous dispersion effect is slight at an absorption band these surfaces are in the general case very closely ellipsoids, with orientations with respect to the crystal axes as required by the crystal system symmetry (see Table IX). When the anomalous dispersion effect is large, an unpolarized beam in the general case on passing through a crystal is not resolved into two plane parallel beams, as by nonabsorbing crystals, but is resolved into two elliptically polarized beams (see Jelley, 1953), and the refractive indices and absorptivities must be determined in accord with these directions of resolution.

The description of the infrared absorption of a crystal may be made in terms of the size, shape, and orientation with respect to the crystallographic axes of the absorptivitrix, and the way these change with wave length. Polarization spectra of a single crystal face, or perhaps even of several crystal faces, may not give all of this information. For a crystal system whose absorptivitrix is fixed with respect to the crystallographic axes, all absorption bands have either a maximum or a minimum value for one optimum orientation of the polarizer. But for a crystal system whose absorptivitrix is not fixed by symmetry requirements with respect to the crystallographic axes, different absorption bands will have their maximum absorptivities at different angles of the polarizer, and polarization spectra of a single crystal face cannot give the maximum dichroic ratios for all absorption bands. The absorptivitrix may be visualized as rotating as well as changing size and shape with change of wave length, and its orientation does not necessarily correspond to that of the indicatrix. In Table IX, a_α, a_β, and a_γ represent the axes of the absorptivitrix, and ϕ_{aa_α}, ϕ_{ba_β}, and ϕ_{ca_γ} represent the angles these axes make with the a, b, and c crystallographic axes respectively. Tabular description of the absorptivitrix would in the general case require six parameters at each wave length if the surface is an elliposid; if the anomalous dispersion effects are "large," the surface is not an ellipsoid and further description is required. Note that the symmetry requirements of Table IX for the absorptivitrix are identical to those for the indicatrix: one may write n's for the a's and θ's for the ϕ's, although the θ's do not necessarily equal the ϕ's. Ambrose *et al.* (1951) give the quantitation for relating polarization spectra to bond directions, with examples. Additional examples are reviewed by Lecomte (1949).

5. CATALOGING OF SPECTRA

Cataloging is essentially a problem in information transfer: one should be able to "ask" the catalog questions and receive answers of maximum usefulness in minimum time. Concerning infrared spectra, two categories of questions are generally asked: 1) what molecule or molecules could produce the given spectrum, and 2) what is the spectrum of a given molecule. An infrared catalog then should establish correlations between molecules and spectra, and ideally should be able to answer questions of both types. Other questions may be asked concerning the functional groups present in a molecule, the procedures of sample preparation, instrument accuracy, bibliography and spectral interpretations, etc. It may be desirable to subdivide the catalog in terms of these different questions, with the molecule name or a serial number the major means of cross reference. We designate molecules to the limits of our structural knowledge, but different catalogs organized to answer question 1 designate the spectra to differing extents and in general leave out a great deal of potentially useful information in the attempt to select the aspects of greatest distinction.

Chemical designation must be complete enough to allow the obtaining of the same data for a sample believed to be the same. It is emphasized that for solids this must include molar proportions of inorganic substances of salts or hydrates (see Clark, 1950a), or crystallographic designations of polymorphs (Kendall, 1953), for if these differ although the parent compound does not, the spectra may differ in major ways. Sequencing may be in the following terms:

1) The atomic formula. Chemical Abstracts uses the "Hill system," with an alphabetical sequence of elements for inorganic samples and a C—H-alphabetical sequence for organic samples. Others use the "Richter system," with the sequence CHONClBrIKSP-alphabetical.

2) The systematic name. Chemical Abstracts uses the alphabetical sequence of the parent compound, with subdivisions in terms of the substitution groups (see Tables VII and VIII).

3) The common name. A user of the catalog may not know the procedures of systematic nomenclature. For molecules of high molecular weight, particularly natural products, the systematic name may be too long and unnecessarily precise for adequate molecular differentiation, or the details of structure may not be known.

4) The functional groups. This sequencing has not been systematized, but a number of the infrared catalogs may be readily searched in terms of functional groups, because of the importance of this point of view in interpreting infrared spectra.

The spectra available in catalogs are in a variety of forms as to axes' scales and as to sample preparation. This generally precludes a direct comparison on a "light box" or a projection device of an experimental spectrum placed over a catalog spectrum or its linear enlargement, and so a more careful representation of spectral differences is required. Van Zandt Williams (1951b) suggests a standard form of two sheets. The ordinates of each are 20 cm and are linear in transmittance, but labelled and with grids in absorbance units, with 0 absorbance at the top. The abscissas are 90 cm long and are linear in wave number, decreasing from left to right, with grid marks every 2 cm. The first sheet has three regions: 9000–4000 cm^{-1} (quartz), at 500 cm^{-1}/2 cm of paper; 4000–2000 cm^{-1} (LiF), at 100 cm^{-1}/2 cm of paper; and 2000–1250 cm^{-1} (CaF_2), at 50 cm^{-1}/2 cm of paper. The second sheet has two regions: 4000–2000 cm^{-1} (LiF, NaCl), at 200 cm^{-1}/2 cm of paper; and 2000–250 cm^{-1} (NaCl, KBr-I, CsBr), at 50 cm^{-1}/2 cm of paper. The Perkin-Elmer Corporation supplies devices for recording such spectra, with automatic gear changes, but prisms must still be changed by hand. These scales may not become standard, but the 4/1 change of grid scale unit at 2000 cm^{-1} is widely used. A common objection to spectra, particularly those published, is that the grids are not fine enough to allow the reading of band positions and intensities to the accuracy of their recording, perhaps 1 to 5 times the pen width. If grids are to be left out, and the spectrum reduced in size, there should be an adjoining table giving band positions and intensities. The author has been moderately successful in double printing fine grid scales onto photographs of published spectra. It is also very useful to have a "sharp" transmission positive (on gelatin or glass plate) of a fine grid. This can be projected, with adjustable enlargement by means of an enlarger in a darkroom, onto a published spectrum to allow its accurate reading. An optical company might be encouraged to supply a lens to convert linear wave length to linear wave number scales.

Catalog makers likewise have variable criteria as to sample purity, which affects particularly the significance of the weaker bands; sample preparation, particularly as to thickness and the band heights; instrument accuracy, as to calibration, noise, and resolution; procedures for "balancing out" sample "scattering"; etc. The significance of these criteria depend on the level of utilization of the spectral information. For example, if a sample is to be identified by six or seven strong bands designated only to the nearest 0.1 μ, with criteria of sample purity determined from other procedures, the catalog spectrum need not be recorded with high resolution nor of a sample of extreme purity. One would prefer to see data thrown away in the utilization of a catalog rather than in its preparation, for the later procedure limits the catalog to the present and generally qualitative procedures.

The notable book catalogs of spectra are Barnes *et al.* (1944), with the spectra of some 360 molecular types, about 25 of which might be found naturally in living organisms; Randall *et al.* (1949), with the spectra of some 390 molecular types, about 65 of which might be found naturally in living organisms, and Landolt-Börnstein (1951), with some 1000 spectra compiled by H. Seidel, with sections on the biochemical categories. The spectra are not of uniform quality, often with omitted spectral regions, and, in some cases, represented by line diagrams. Barnes *et al.* (1944), index their spectra and 2701 literature references in terms of broad molecular categories, atomic formulae, and common names. Randall *et al.* (1949), index their spectra by categories of molecular types and by common names, and by wave length for the region 4.8 to 6.9 μ. They also give an index by common name to references and an index by wave length to spectra of some 130 theoretically analysed molecules, but these spectra are not all presented. The rotational and vibrational spectra indices in Landolt-Börnstein (1951), compiled by Maier, Mecke, Kerkhof, Pajenkamp, and Seidel are sequenced by atomic formula, with cross references to the spectra and Beilstein, and with 2394 literature references into 1949 in volume I, part 2, and 565 references through 1950 in volume I, part 3, on Raman, infrared, microwave, and theoretical studies. Mecke and Kerkhof also give indices in sequence of molecule point group symmetries to the fundamental vibrations of over 600 compounds. Other catalogs of infrared spectra are on separate sheets or cards, and may be arranged and resorted as desired.

The "API" Research Project 44 Catalog of Infrared Spectrograms (American Petroleum Institute, 50 West 50th Street, New York, N. Y.), under the direction of Dr. F. D. Rossini, of Carnegie Institute of Technology, is published on loose leaf notebook sheets. Some 1500 spectra of about 1000 compounds, particularly hydrocarbons, are available. These are indexed by molecular categories, further subdivided by atomic formulae, and by serial numbers. They have also been indexed on IBM cards as to band positions, molecular structure, etc., according to the Kuentzel system (see below). Another hand sorted system is that of Clark (1950b). This system is primarily directed to the identification of unknown spectra, but in its application to some 85 pyrimidines, purines, nucleosides, nucleotides, and nucleic acids (Clark, 1950a) the catalog is further indexed by technical name and common name, atomic formulae, and structural formulae, with cross references to the Chemical Abstracts fourth decennial index, Heilbron *et al.* (1943) Dictionary of Organic Compounds, and Beilstein. This system utilizes a three by five inch card. The three strongest bands in the region 9–15 μ (1111 − 667 cm^{-1}) are the index key, in analog to the x-ray catalog system of Hanawalt *et al.* (1938), with the cards being filed in sequence of the position of the strongest band. "Intensities" are given in terms of absorbance ratios of all the bands to that of the strongest in the 9–15 μ region. Note if the absorption law applies and there is an adequate correction for "scattering", by means of the empirically drawn base line, that absorbance ratios equal absorptivity ratios and are characteristic physical constants of the sample, being independent of concentration or thickness. This hand sorted catalog may be searched to the reproducibility-accuracy limits of the recording instrument and operator, 0.02 to 0.04 μ, and so one can more rapidly identify a spectrum from a catalog of up to perhaps 1000 spectra than by keysort or machine sorted catalogs on which the spectra are punched with poorer resolution, commonly to 0.1 μ or worse. This system is particularly recommended for use by laboratories making studies of a limited number of spectra prior to their incorporation into the national catalog. This system also makes use of band intensities, an aspect commonly neglected by other systems. The author has the impression that catalogs might be more efficiently machine sorted by a detailed punching of band positions in the "fingerprint" region than by a coarser punching of band positions throughout the spectrum.

The first commercially available keysorted catalog system was that of Samuel P. Sadtler and Son, Inc., 2100 Arch Street, Philadelphia 3, Pennsylvania. Full size Baird instrument spectra are lithographed on large cards the margins of which are notched into the marginal holes as to band positions, functional groups, etc. The catalog is searched by pushing one or several needles through the holes of the spectra lined up on edge. When the needles are lifted, only those cards notched at all needle positions will fall through. This system is moderately unwieldy due to the size of the cards, and is now reindexed on IBM cards according to the Kuentzel system. The cards cost from 30 cents each, for complete subscriptions by universities, to $1.25 each for special orders; for current prices and an index of available spectra (some 5000) write Philip Sadtler.

The need for a national, or preferably international, catalog of spectra was recognized, and several years ago a committee was organized at the Ohio State Molecular Structure Symposium, with E. Carroll Creitz as chairman. Thomas V. Parke organized a group to systematically review the "infrared" literature, sending abstracts to Mr. Creitz and the data required for the Kuentzel IBM card index system to Dr. Kuentzel. At present 41 volunteers abstract articles concerning uses of infrared radiations appearing in 29 journals, these containing some 90% of such articles published in the western half of the world. Additional volunteers are requested to increase this coverage; please write Mr. E. Carroll Creitz, National Bureau of Standards, Washington 25, D. C. This program now has the support of the American Society for Testing Materials, the National Research Council, and the National Bureau of Standards. The IBM cards are to be made available by the A.S.T.M., at probably less than one cent a card, and the abstracts by NRC-NBS. The NRC-NBS also are making available the NBS infrared files, both literature abstracts and compound cards, many of which will have spectra of the compound either as recorded at the Bureau or as especially submitted to it for inclusion in the file. The bibliography file may be obtained on typewriter paper or on keysort cards, punched has to author, journal, and subject. The compound file may be obtained on typewriter paper or on keysort cards punched as to infrared band positions, functional groups, etc. The spectrum is shown on the back, in some cases with a table of band positions. Some 275 compound cards, at about 10 cents each, and 250 bibliography cards, at about 5 cents each, are now available. The compound file at the National Bureau of Standards contains some 11,000 compounds, so that we may hope that before very long we will have a national infrared catalog in being, now that the procedures for its publication and growth have been established. To obtain these cards, write Mr. Creitz. It is reasonable to expect that in a few years one may make reference by serial numbers to "known" spectra in the national catalog, requiring the publication of only the "unknown" spectra in research reports concerning identification by infrared spectra.

Keysort catalogs take too long to search when there are several thousand cards. It became obvious to a number of industrial groups, who were the first to have such large catalogs, that more mechanized methods of searching were desirable. The system developed by Dr. Lester E. Kuentzel, of the Wyandotte Chemical Company, Wyandotte, Michigan (Kuentzel, 1951) has been adopted by the American Society for Testing Materials, and is now widely used as the national catalog index method. This system uses standard 3¼ by 7⅜ inch International Business Machines (Hollerith) punch cards, each of which has 80 columns, with 12 rows per column: y and x overpunches then 0 to 9. This gives sufficient room to punch in the spectral positions of all absorption bands with transmittances less than 80% of the adjacent base line, the chemical structure as to atoms, unsaturation, chain length, rings, ring substitutions, and functional groups in considerable detail, physical state, melting or boiling point, a source or literature reference, a serial number, and a few other columns for individual use. The form of this card is copyrighted so that all users will maintain the same key, allowing the ready interchange of cards. The cards may be punched by a hand operated device, or by an IBM punching machine. They can be copied automatically by an IBM copying machine, and so can be considered expendable, costing less than one cent each. They may be sorted for one or more punches one column at a time by IBM sorting machines at rates from 250 to 650 cards per minute depending on the model used, 0-80 or 0-82. Sorting efficiency improves with experience, as to which position should be examined first. "Negative" sorting, for all cards which do not have a given punch, may be useful. Using the moderately similar automatic punch card system of Dr. Norman Wright, Baker *et al.*, (1953) find that on the average 11.9% of their file is isolated by sorting at just one spectral position. They emphasize the advantages of sorting the cards by means of an IBM collator, 0-77, which examines several columns simultaneously, leading to more rapid identifications, particularly of spectra of mixtures. The IBM cards do not show the spectra or name the compounds directly; these must be looked up by means of serial numbers printed on the cards. Note, however, that the crude card representation of the spectrum in terms of the wave length positions to the nearest tenth of a micron of the ten or so strongest bands is adequate for a unique qualitative identification of a pure compound or of the major components of a simple mixture from a catalog of several thousand compounds; the complete spectrum contains extra information which may not be necessary for the solution of many infrared problems. Dr. Kuentzel is also developing IBM card indices for x-ray diffraction, ultraviolet absorption, visible absorption, mass spectrographic, and raman data, and bibliography. These indices ideally will refer by serial numbers to a common pool of all chemical and physical data for each compound. Establishing and making inclusive such pools should also become a national (international) project, for one is but partially benefited in using an IBM card index in ten minutes which only refers to scattered original literature which may take days to assemble. It may be desirable that the serial numbers not be arbitrary but that they be the Beilstein system numbers or, better, the Dyson code numbers. Certain work along these lines has commenced. It is suggested that incorporation of data into these pools should be the equivalent of their publication, and should be preferred to their publication in the standard journals, which would maintain all present functions except the publication of standard data. Similar ideas have been expressed by Dr. Wallace Brode. For further information, write to Dr. Kuentzel or to the American Society for Testing Materials, 1916 Race Street, Philadelphia 3, Pennsylvania.

As to future developments, one might make a removable tab containing a microfilm of the standard spectrum and other standard data which could be attached to the IBM card, eliminating the time to look up the data by means of the serial number. Preliminary sorting would be by means of the IBM card, but final identification, perhaps even made quantitative, would be by means of an optical device which would compare the experimental spectrum to one or several standard spectra. As suggested in Section II 6e, when a suitable spectrum is to be recorded, as judged from an auxiliary oscilloscope presentation, the infrared spectrophotometer might be automatically coupled to punching and sorting equipment, so that the spectrum and its identification might be completed together. For quantitative work, a more complete digital representation of the spectrum will be useful, perhaps in terms of absorptivity values per unit spectral slit width. Such data might be cataloged on wire or tape, for these do not have the limited information content of the IBM cards. Machines for automatically scanning and sorting such data are already available from International Business Machines, Incorporated, and are used by the Census Bureau. A further step would be an electronic memory or catalog, also involving devices already available, which could be reviewed in seconds. One such device might be utilized by many laboratories by telephone or radio connections. It is emphasized that as the inclusive point of view develops in science, automatic equipment becomes increasingly essential. University or industrial laboratories which do not have automatic sorting equipment are jeopardizing their use of the large amounts of data available, and are jeopardizing their science.

IV. Applications

1. Introduction

This discussion of the applications of infrared spectroscopy to biology should ideally include a microfilm appendix of the spectra of the major biochemicals. The author is working on such an appendix, although the national catalog program may make this unnecessary. It is still often easier to obtain a sample of a biochemical and record its spectrum than to find its spectrum in the literature, and original standard spectra may be desirable in any case because of differences of recording, sample preparation, etc. Catalog spectra are of principal use for preliminary identifications and evaluations of the possibilities of analyses. At present, Landolt-Börnstein (1951) is the best single generally available source for references to spectra of particular molecules, including biochemicals. It is expected that the NRC-NBS national catalog program will soon be more inclusive. Other catalogs should be examined. The *Analytical Chemistry* January reviews of infrared data (1949–52, 1954, ...) are helpful. The subject indices of many journals may be profitably examined under "infrared" or "spectra," although most journals do not yet contain inclusive "physical data" indices and so many do not contain such cross-indexing for articles which include infrared spectra but have different subject emphases. These general sources will not be repeatedly referred to in the discussion below. This discussion is fragmentary; the purpose is to indicate a few of the types of interpretations and uses of biochemical spectra.

2. Biochemical Spectra

a. *Alkaloids, Antibiotics, and Drugs*

The detailed structures of many of these substances are not known. Infrared spectra may be used for identification (Pleat *et al.*, 1951), or with prepared mixtures of known components for quantitation (Washburn, 1953). The large number of absorption bands are of aid in the unique identification of these molecules, but the lack of "sharp" bands not close to other bands makes quantitation more difficult. Using the characteristic wave number assignments of biochemical groups, illustrated in figure 12, the spectra may be used for structural arguments (Janot *et al.*, 1951). The work on "The Chemistry of Penicillin" included many contributions to this point of view (Thompson *et al.*, 1949). Theoretical studies are not yet sufficiently developed to allow a detailed prediction of structure from spectra; the spectra are of greatest use for sensitively indicating identity of synthetic substances of chemically assigned structure and fractions and derivatives of the unknown substance (Balenović *et al.*, 1952; Gore and Petersen, 1949). In certain cases, the biological activity of a substance may be correlated with the presence of a biochemical group which has a characteristic absorption band, so that purification of the substance or the prediction of the activity of a fraction may be made by quantitative studies at this spectral position (Papineau-Couture and Burley, 1952).

b. Amino Acids and Proteins

This subject has recently been reviewed by Sutherland (1952). See also the discussions by Bellamy (1954) and by Thompson *et al.* (1950). Because of the lack of nonaqueous amino acid solvents suitable for infrared studies, Schiedt and Reinwein (1952) devised a "pressed disk" sample method (see Section II 4). Gore *et al.* (1949) studied aqueous solutions. In the 6 μ region, D_2O is more transparent than H_2O. In dilute HCl, the C═O vibration of the un-ionized carboxyl group of an amino acid is at 1740 cm^{-1}, but in water or dilute alkali, bands near 1600 and 1400 cm^{-1} of the ionized carboxyl group, $—C(O)O^-$, are observed. These latter bands are observed in the crystalline amino acids and with the absence of the "free" primary amine bands in the region 3500–3200 cm^{-1}, support the assignment of the zwitterion structure, $RCH(NH_3)^+C(O)O^-$, for the solid amino acids. Other bands may be assigned or named as follows (see Randall *et al.*, 1949): 3050 cm^{-1} (bonded N—H stretch); 2907 (C—H stretch); a series of moderately weak bands between 3.45 and 4.0 μ (bonded N—H or O——H stretch?); 2080 (bonded N—H?); 1620 (Randall's "amino acid I"); 1592 (ionized carboxyl, $—C(O)O^-$; a shifted C═O stretch); 1510 (Randall's "amino acid II"; N—H bend); 1455 (C—H bend); 1412 (ionized carboxyl; a shifted C—O stretch); and 1359 (C—H bend). Bands closely similar in position are commonly found for solid amino acids. Bands at longer wave lengths are more affected by structural modifications, and hence are more useful for identification purposes. When the amino acids are combined as peptides or proteins, these bands are modified, so that it is in general not possible to determine the amino acids of a protein from the protein spectrum. In special cases and to limited extents, this may be possible (Astbury *et al.*, 1948), but in general it is necessary to partially hydrolyse the protein before the spectra of the pieces allow unique identifications. At present one must hydrolyse all peptide linkages before the spectra are recognized (Darmon *et al.*, 1948), but the simpler peptides have specific spectra (see Thompson *et al.*, 1950, for line diagrams of the spectra of di, tri, tetra, penta, and polyglycine). As these spectra become catalogued and made inclusive for all the naturally occurring shorter sequences and combinations, of amino acids or other molecules, which can be specifically identified by their spectra, one may expect that infrared spectra of partial hydrolysis products will become an important tool in determining the structures of proteins or other macromolecules. Hurd *et al.* (1953) further illustrate the specificity of spectra of synthetic polypeptides.

Polypeptides evidently have but a few stable configurations of the "backbone" (Corey and Pauling, 1953): the fully extended β configurations, with the peptide C═O and N—H bonds perpendicular to the chain length, and perhaps a few folded α configurations. Not only are the bonds differently oriented for these different types, but the cross linkages (hydrogen bonds) between the chains are different. Hence the different configurations will have different dichroisms, as observed with polarized infrared radiation, and slightly different spectral positions of those bands affected by hydrogen bonding (Elliott and Ambrose, 1950). Proteins commonly have the following absorption bands: 1.5-2.4 μ (several overtone bands of N—H, C—H, and C═O vibrations, which may be useful for near infrared microspectrophotometry; see Bath and Ellis, 1941); 3310 cm^{-1} (H bonded N—H; slight α,β differences); 3060 (H bonded N—H and aromatic C—H; α weak parallel dichroism, i.e. for proteins with α configurations the absorbance is slightly greater with the electric vector of the radiation parallel to the chain length, β weak perpendicular dichroism); about 2900, broad and multiple bands (C—H); 1650 − α, strong parallel dichroism, *or* 1630 − β, weak perpendicular dichroism (peptide C═O); 1575 (ionized carboxyl C═O; this band is weak and may not be resolved); 1550 − α, perpendicular dichroism, *or* 1530 − β, parallel dichroism (peptide N—H bend); 1450 (C—H bend); 1400 (ionized carboxyl C—O, weak band); 1380 (C—H bend; this may be absent); 1230 (unassigned); 1160 (N—H bend?); 1070 (unassigned; this is not as constant in position as the other bands. In tissues it may be contributed to by vibrations of nucleic acids and other substances); and 720 (unassigned; a broad band). Note that the dichroism gives a measure of the orientations of the vibrations and hence of the bonds. Protein folding hypotheses may be evaluated by studies of polarized infrared spectra (Price and Fraser, 1953). Different proteins may have a few other bands, and slightly different band intensities, but their broad banded spectra are far from adequate to characterize the degrees of possible structural variation. It is not until the hydrolysis fragments have molecular weights below about 300 that the spectra allow unique characterizations of structure, although this limit may be extended upward by a careful study of band intensities as well as positions. Effects on spectra of denaturation and of pH changes in solutions are described by Lenormant (1953).

c. Carbohydrates.

Kuhn (1950) gives the spectra of 79 carbohydrates, indicating the specificity of identification possible. The bands near 3 μ are due to the bonded

O—H groups. The 3.4 μ band is due to saturated C—H vibrations, particularly of the mineral oil mulling agent. Carbohydrates generally are characterized by having one or several strong if not strongest absorption bands between 9 and 10 μ, attributed to saturated ring and chain C—C and C—O stretching vibrations. Whistler and House (1953) further demonstrate spectral differences between α and β sugars. They are only partially successful in correlating particular bands with the α or β configuration. When inclusive catalogs are available, such correlations will be necessary only for analyses of newly isolated carbohydrates. Kuhn (1950) determines the degree of nitration of celluloses by the relative intensity of the remaining O—H to C—H bands. Thompson *et al.* (1950) also give the spectra of cellulose derivatives. Darmon and Rudall (1950) report on chitan and derivatives, using polarized radiations, and Orr *et al.*,(1952) report on hyaluronic acid, chondroitin sulfuric acid, and derivatives. As with the proteins, it may be necessary to cleave these polysaccharides before the spectra allow unique structural characterizations, although it may be that in nature there are few enough macromolecule structures, without intermediate variations, that a detailed study of band intensities as well as positions will allow a distinction of the major types. Friedel and Pelipetz (1953) compared the spectra of carbohydrate chars and coal, and indicate structural relations.

d. Hormones and Vitamins

The major studies by Dobriner and coworkers, reviewed by Jones and Dobriner (1949), of urinary steroids isolated by extraction and chromatography demonstrate the sensitive characterization of steroid structures possible by infrared techniques, a characterization made with difficulty by chemical methods. Due to the basic similarities of the "skeletons" of the natural steroids and the unique spectral characteristics of the natural substitution groups, the characterization can be complete as to steric structure by comparison of the spectra to those of steroids of known structure in spite of the fact that some of these molecules have molecular weights approaching 400. Infrared spectra have become a major tool in problems of steroid chemistry (Voser *et al.*, 1952). Steroid glycosides and the protein hormones may not be adequately characterized without breaking the molecules. Vitamins may be well characterized by infrared spectra. Rosenkrantz (1948) describes the spectra of the vitamin E group. Trotter *et al.* (1948) describe the potentialities of vitamin C analysis by infrared spectra, for colorimetric methods may have inadequate specificity. On the isolation of a new vitamin, infrared spectra should be but one of many means of its characterization which are reported (Fantes *et al.*, 1949). An atlas of steroid spectra is now available (Dobriner *et al.*, 1953).

e. *Inorganic substances and miscellaneous metabolites.*

Miller and Wilkins (1953) present the spectra of 159 inorganic substances, which contain 33 polyatomic ions. These ions have characteristic frequencies, but to distinguish closely related inorganic samples the spectra may have to be examined carefully for slight shifts of band positions and intensities. The smaller metabolites may be well characterized by their spectra. Fowler (1949) has utilized the infrared absorption of carbon dioxide in a study of respiration. Vonderwahl and Schinz (1952) utilize infrared spectra of synthetic derivatives in their chemical study of the odor of violets. Kudzin and Nord (1951) utilize infrared spectra in their interpretations of the structures of wood lignins. Infrared spectrophotometry is becoming a major tool of the synthetic and analytical chemist, as a means of rapidly identifying the products of his reactions.

f. *Lipids and Derivatives*

The long chain fatty acids, their methyl esters, their alcohol forms, and triglycerides may be distinguished from each other, but within each class the spectra must be examined with care to distinguish the different members if indeed this is possible, for changes of chain length do not markedly modify the spectra (Shreve *et al.*, 1950). Long chain compounds with a *trans* monounsaturation characteristically have a band at 10.36 μ (965 cm^{-1}), probably related to C—H bending vibrations at the unsaturation position, which is not found in the *cis* or saturated compounds. The *cis* unsaturated compounds are not as uniquely distinguished, although they appear to have greater absorption in the 13.8–14.3 μ region relative to the C=O band near 5.8 μ than the *trans* compounds have (Shreve *et al.*, 1950). Sinclair *et al.* (1952) find that solid state spectra, with the sample crystallized at liquid nitrogen temperature if necessary, have more detail and allow better quantitation. A branching in the chain of the fatty acids may be distinguished if within five carbon atoms of the carboxyl group (Freeman, 1952). Published spectra of the lecithins appear identical (Baer, 1953); enzymatic or chemical degradation will be necessary for their detailed characterization by infrared spectra. Infrared studies of macromolecules should be parallelled by x-ray diffraction, electron microscopy and other work, for each field gives a different partial evidence. Thus phthiocerane (molecular weight 378), the hydrocarbon derived from the wax alcohol phthiocerol, has an infrared spectrum which is apparently identical with those of 4-methyl tritriacontane and 4-methyl tetratriacontane, but different from the spectra of the 2-, 3-, or 5-methyl derivatives. X-ray diffraction and melting point evidence favor the 4-methyl tritriacontane assignment (Ställberg-Stenhagen *et al.*, 1947).

g. Nucleic Acids and Derivatives

The infrared spectra and x-ray powder patterns of 85 nucleic acid type derivatives were described by Clark (1950a). This work is available on microfilm and so has not been published elsewhere, a procedure now recognized as inadequate. The derivatives are well characterized by their spectra (see also Blout and Fields, 1950). These may be used to evaluate the contributions of the various resonance and tautomeric forms of these structures (see Fig. 11), particularly in comparison with spectra of *N*-methylated derivatives which cannot undergo this tautomerism and of deuterated derivatives (Short and Thompson, 1952; Brown and Short, 1953). Keto and amino forms predominate over the enol and imino forms, but it is emphasized that this is not an all or none situation; all tautomeric forms may be present within the course of a measurement, each with its spectrum. The different tautomers will have different electronic structures, as represented by the stylized resonance diagrams, with different force constants and internuclear distances. This work should be paralleled by single crystal x-ray diffraction studies, which give the averaged internuclear distances. Umberger and Adams (1952) present the spectra of 20 barbitruates. Lacher *et al.* (1949) studied near infrared spectra of three pyrimidines; these bands may be useful in microspectrophotometric studies. As examples of the specificity of identification by both infrared and x-ray techniques, yeast adenylic acid, which is probably a mixture of adenosine-2′- and adenosine-3′-phosphate, is readily distinguished from adenosine-5′-phosphate; deoxyriboguanosine from (ribo) guanosine; adenine from adenine hydrochloride and from adenine sulfate; uridine (1-ᴅ-ribosyl-uracil) from 1-ᴅ-arabinosyl-uracil and from 1-ᴅ-glucosyl-uracil; etc. (Clark, 1950a). With the polynucleotides, the spectral detail is lost, although deoxyribonucleic acids can be distinguished as a group from ribonucleic acids (Blout and Fields, 1949) by slight differences in band positions and notably with a band near 9.8 μ of DNA which is not found with RNA (see Fraser, 1953, and Fraser and Chayen, 1950). Using polarized infrared radiations, Fraser and Fraser (1951) conclude that the purine and pyrimidine bases are approximately perpendicular to the chain axis or direction of smearing with a DNA sample.

h. Pigments

Chlorophyll a, with a methyl group at C3, and chlorophyll b, with a formyl group at C3, are readily distinguished by their infrared spectra, although their molecular weights are near 900. They are also distinguishable by spectra from pheophytin a and bacteriochlorophyll a, for these molecules differ by spectrally distinctive groups. (Weigl and Livingston, 1953). It is expected that synthetic derivatives with molecular weights this high and with less significant structural differences might not be uniquely identified by their spectra, but if nature's economy affords only a few distinctive types, the spectra can uniquely identify the natural substances, although spectra of degradation products of a newly isolated substance should be examined to insure that a minor structural variation had not been found. This same reasoning applies to other macromolecules: if there are but limited numbers of natural types of oligopeptides or oligonucleotides, for example, the infrared spectra may be adequate for unique identifications although the molecular weights go into the thousands. Granick *et al.* (1953) present the spectra of hematoporphyrins. Weiss and Nord (1949) utilize infrared spectra as guides in the preparation of synthetic analogs of a mold pigment, solanione.

3. Infrared Spectra of Cells and Tissues

Biological cells are complex aggregates of perhaps thousands of molecular types, all within the few microns span of this remarkable unit of organization. In spite of an internal viscosity commonly not many times that of water, it appears a better approximation to think of cells as "crystalline" in terms of the precision of their molecular organization than as a random mixture of these molecules, for how else could the thousands of specific chemical reactions be carried out in proper sequence? Cytology is the study of this organization, and the classical work provides a vast storehouse of information as to distinguishable differences within cells as already posed problems requiring biochemical and physiological interpretations. Cytochemistry is the attempt to fit biochemistry into the cell. The cell is so small, mammalian cells weighing perhaps 10^{-9} grams, that this seems a tight fit; I find it useful to employ a mental magnification of 10^7, at which one angstrom becomes one millimeter and molecules may be bent out of wire yet the microscopically seen cell components may still be visualized. This description of cells and cell functions at both microscopic and molecular levels I call "molecular cytology," and the program is to learn the properties of molecules and the ways they orient and interact with other molecules, the inclusive identification and localization of molecules in cells, and the properties of cells, that these molecular cytology descriptions may be established and interpreted (see Clark, 1952). Physical methods of chemical identification, following systematic fractionation by extraction, centrifugation, hydrolysis, chromatography etc., offer the means for such inclusive biochemical analyses. When the complexity of living organization is recognized, it is remarkable how few variables are simultaneously measured in most biological studies. It is expected that the inclusive structural biochemical descriptions of molecular cytology made with any biological study will provide a measure of the "constancy" of the biological system and indicate interpretations of the aspects of inconstancy.

Spectra, fig. 14, provide a means of beginning such descriptions. The

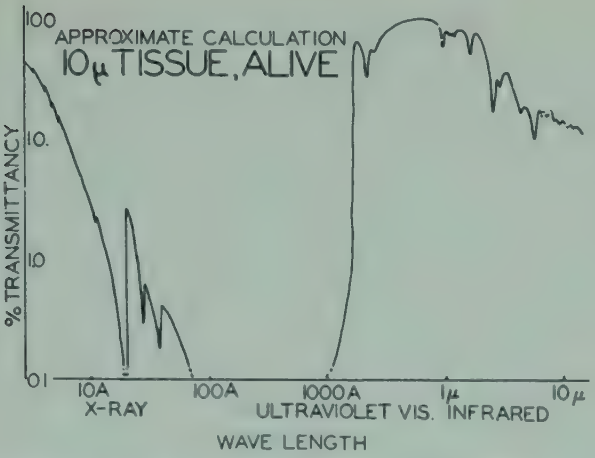


FIG. 14. A calculated tissue spectrum. (From Clark, 1952, *Applied Spectroscopy* **6,** 1, courtesy of the editor.)

x-ray absorption bands, studied notably by Engström (1946), provide a measure of atomic concentrations. Uses of the ultraviolet absorption bands, near 0.26 and 0.28 μ, of the unsaturated groups of nucleic acids and proteins, are described in Chapter 4 of this book. The infrared absorption bands near 0.97, 1.9, 3, 4.7, and 6.1 μ are due to water, which makes up some 75 % of the mass of mammalian soft tissue cells. Other bands are principally due to proteins, with lesser effects due to lipids, carbohydrates in some cells, and perhaps nucleic acids, although these make up only about 1 % of the soft tissue wet mass. Different cells have different cytological structures, the bulk of our information, and hence different chemistries, with only preliminary evaluations. Small molecules other than water and inorganic substances make up perhaps only 1 % of the wet mass.

Tissue spectra have inadequate detail to allow the evaluation of all molecular types, or "degrees of freedom" of possible chemical variation. Each of the broad bands are contributed to by many molecular types, although it may be found that certain bands are largely due to a category of molecular types. The 6.5 μ band (principally N—H bend), for example, may prove as useful as the 0.28 μ band for the evaluation of total tissue proteins. Different tissues have slightly to moderately different spectra, which may be reproducible and empirically useful, but the evaluation of the chemical differences by this means alone can only be in terms of broad possible categories.

The spectrum is an "averaged" absorption, and scattering, of all substances within the beam of the spectrophotometer. Since the cell is chemically heterogeneous and adjacent cells may differ in structure (including chemistry) and function, this "average" may apply exactly to no natural combination of molecules, a limitation shared by biochemical analyses of the necessarily pooled components of millions or billions of cells. However, these analyses provide a first approximation of cell chemistry. A microscope reduces the beam size (see Section II 6d), but with present devices, one dimension of the sample must be near 100 μ to obtain satisfactory spectra. Moreover, the minimum volume to which the absorption can apply, set by the resolving limit of the optics, has a diameter of the order of the wave length of the radiation used. For this reason and the usability of glass or quartz optics and photoconductive detectors, and the greater transmission of water, it is expected that near infrared microspectrophotometry of tissues, of the overtone absorption bands, may be as useful as studies at the longer wave lengths. Infrared spectra of intact cells can be used to the specificity of the method as an indication of the similarity of different groups of cells but cannot be used for any detailed identification and localization of the structural biochemistry of cells.

Blout and Lenormant (1953) have studied living bacteria. Wood (1951) studied living muscle cells, and reports variations of relative band intensities, particularly at 9.7 μ, for different muscles of the frog and for different species. Spectra of 1 % ATP could not be used to characterize this substance, and Wood concludes that substances making up less than 1 % of the tissue probably cannot be detected by the spectra. The "fixed" biological cell is essentially a protein and nucleoprotein skeleton. Spectra of dehydrated material show a few broad bands largely due to protein. Different tissues have different spectra. For example blood smears have two bands at 8.55 and 9.05–9.15 μ which are not seen in the fixed tissue spectra, and the fixed tissue band at 9.20–9.35 μ is not seen in blood spectra (Blout and Mellors, 1949). Different organs have different spectra; brain tissue spectra are modified by insulin shock treatment (Schwarz *et al.*, 1951). Cancerous tissue spectra differ from normal (Woernley, 1952). Blood pathology may be indicated by spectra (Lenormant, 1951; Agnew *et al.*, 1952). Different species of bacteria may be characterized by their spectra (Stevenson and Bolduan, 1952), but crude preparations of different viruses give spectra which are too variable for identification purposes, although infected preparations may be distinguished from uninfected (Benedict *et al.*, 1954). Infrared spectra of algal flagella aid in their characterization as having the α keratin structure (Astbury and Saha, 1953). Morales and Cecchini (1951) report slight relative intensity differences at 8.33 and 9.75 μ of spectra of lyophylized resting and contracted muscle. Anomalous dispersion effects at the different absorption bands have not been evaluated, but these certainly could modify relative intensities of the different absorption bands, an effect which is not corrected for by the usual procedure of recording the spectra with a roughened salt plate in the reference beam as an approximate correction for scattering. Part of the radiation loss as the beam passes through the sample is due to selective scattering (anomalous dispersion) as well as to selective absorption. Adequate evaluation of this effect and perhaps even any quantitative measurements with samples of this type will require a goniometric spectrophotometer with which the scattered radiation can be directly measured (Clark *et al.*, 1953).

Infrared spectra, and spectra at other wave lengths, of adjacent regions of a tissue can give a rough measure of tissue homogeneity, and should be systematically utilized as a control of this important factor, but fractionation and other procedures must be utilized to obtain detailed chemical identifications. Fraser and Chayen (1952) show that ribonuclease reduces but does not completely remove the absorption bands of bean root tip at 997, 969, 916, and 860 cm^{-1} (10.03, 10.31, 10.91, and 11.62 μ), bands also shown by RNA, and that extraction with N/10 HCl reduces the absorption bands of sperm at 967, 830, and 895 cm^{-1} (10.34, 10.75, and 11.17 μ), bands also shown by DNA. They suggest these methods for the determination of these nucleic acids, but do not yet illustrate the far more difficult analysis of both nucleic acids in less favorable material. Note also that these analyses cannot provide localizations within the cell. Levine *et al.* (1953) grew enteric bacteria on nutrient agar with glucose, and found absorption bands of dried films of the bacteria at 8.7, 9.25, and 9.75 μ. Without glucose in the medium, spectra of these bacteria only had the band at 9.25 μ. Aqueous extraction, centrifugation, and precipitation, using the former bacteria, led to the isolation and identification of glycogen; they conclude that under certain conditions the 9.75 μ (1026 cm^{-1}) band of whole cells may be used as a measure of glycogen content, with the 6.45 μ (1550 cm^{-1}) protein band as an internal standard. A further study of bacteria is reported by Randall and Smith (1953), utilizing solubility and chromatography fractionation. The spectra of the fractions, not chemically specified in detail, provide means of evaluating the reproducibility of techniques of operations, of distinguishing between virulent and avirulent strains of tubercle bacilli and between human and bovine varieties, and even of distinguishing between different strains of tubercle bacilli from patients ill with the "same" disease, pulmonary tuberculosis. The spectra of the fractions, which can be reproducibly obtained with different batches of the same bacillus sample, are more detailed than the spectra of the intact bacteria, and so allow more detailed distinctions between types to be made.

An extension of these methods is the procedure to obtain the inclusive biochemical analyses of "molecular cytology." The utilization of automatic equipment will determine the degree of this inclusiveness, and eliminate the too frequent notations "fractions discarded" in present studies of cells. One can visualize an automatic continuous flow device into which one puts a sample, which at appropriate steps in extractions, grinding, centrifugation, hydrolyses, chromatography, etc. records, identifies to the extent of the associated memory circuits, and totals the infrared spectra of the fractions, to provide inclusive biochemical analyses of at least the hydrolysed small molecule components, and with localizations determined by the initial procedures of cell fractionation. This device is possible by present technology, and should be available to and utilized by biologists, and chemists, in many lines of work. If the chemistry of a sample can be identified by machines, there is no longer a reason for this chemistry to be a mystery to man.

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Chapter 6

THE LIGHT MICROSCOPE

L. C. Martin

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I. The Principles of Lenses and Optical Systems

1. THE CONCEPT OF THE RAY

The shadow of an opaque object which screens a part of a plane surface from a very small source of light corresponds to the geometrical projection of the object upon the plane, so that the boundary of the shadow is intersected by lines from the source tangential to the surface of the object. This phenomenon is consistent with the supposition that the rays of light, which are the lines of energy-flow from the source, travel outwards in straight lines in all directions. Such a shadow has some of the properties of an "image"; it is a geometrical figure from which some partial information can be gained as to the shape of the corresponding object.

The pinhole camera, Fig. 1, restricts rays reaching a plane screen, and admits only those which pass through the small aperture. A three-dimensional array of small sources of light will thus produce an 'image' on the plane screen, the image being a perspective projection of the objects with respect to the pinhole. If the object-array is, say, a landscape, the image provides a "picture" in which distributions of color, light and shade, etc are interpreted, when seen, as trees, houses, etc. Experience then contributes greatly towards the assessment of the three-dimensional relations; but if the object-array is completely unfamiliar, interpretation of the space relations presents great difficulties. However, if it is known that the objects are contained in one plane parallel to the image plane, the geometrical conformations of the image are similar to those of the object. In other words a plane object-pattern of color and brightness will be (with certain limitations) reproduced as a corresponding plane image pattern. If h is the distance between two points in the object plane, and h' the corresponding distance between corresponding points in the image plane, then the "magnification" is given by

$$\text{Magnification} = \frac{h'}{h} = \frac{l'}{l}, \tag{1}$$

where l and l' are the perpendicular distances from the pinhole to the object and image planes, respectively.

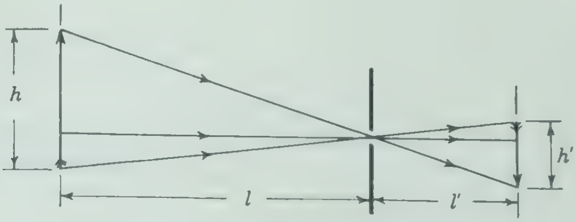


FIG. 1. The Pinhole Camera.

2. Reflection, Refraction, and Dispersion

Rays of light suffer abrupt changes of direction when they encounter a surface separating one transparent medium from another. Some of the light is *reflected* back into the same medium, while some is *refracted* through the boundary. (See Fig. 2.)

The laws of reflection and refraction for monochromatic light must be given an exact statement, but two preliminary definitions are necessary.

The *normal* at a point in a surface is the straight line through the point, drawn perpendicular to the tangent plane touching the surface at this point.

The *plane of incidence* of a ray meeting the surface is the plane containing the ray and the normal to the surface at the point of incidence.

The *law of reflection* states that (a) the acute angles made by the incident and reflected rays respectively with the normal at the point of incidence are numerically equal; and (b) the reflected ray remains in the plane of incidence, but is situated on the opposite side of the normal.

The law of reflection applies to any truly reflecting surface, transparent or opaque.

The *law of refraction* states that (a) the ratio of the sines of the acute angles made by the incident and refracted rays respectively with the normal at the point of incidence is a constant, characteristic of the combined action of the two media; and (b) the refracted ray in the second medium remains in the plane of incidence, having crossed the normal at the point of entry.

A homogeneous medium has, in fact, a specific "refractive index"; and it is the ratio of the refractive indices which determines the ratio mentioned in the definition. Thus if for practical purposes the refractive index of air is unity, that of water for yellow monochromatic light is about 1.33, of crown glass is about 1.52, of flint glass about 1.65. If n is the refractive index of the first medium, while n' is that of the medium receiving the refracted ray, while i and i' are the acute angles of incidence and refraction,

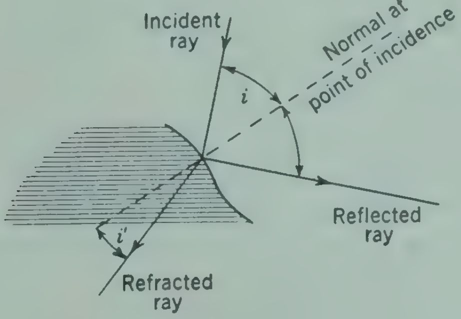


Fig. 2

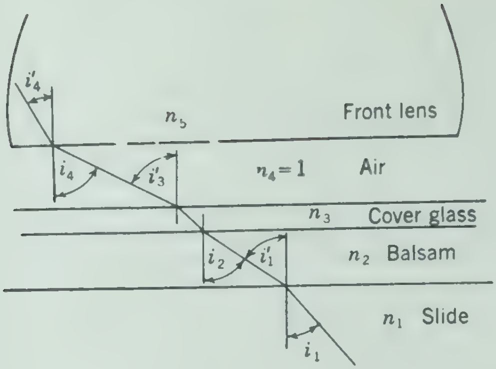


Fig. 3

the first part of the law can be put into the algebraical form

$$n \sin i = n' \sin i' \tag{2}$$

Consider for example a ray of light passing through a series of parallel layers of different materials, Fig. 3, as in the case of a microscope slide supporting a layer of Canada balsam covered in its turn by a cover glass separated from the parallel front surface of a microscope objective by a parallel layer of air. The refraction from the slide to the balsam is represented by

$$n_1 \sin i_1 = n_2 \sin i_2 ,$$

and that from the balsam to the cover glass by

$$n_2 \sin i_2 = n_3 \sin i_3 ,$$

and so on, so that the product of the refractive index and the sine of the angle of obliquity is constant for all the layers.

Note, however, that when the layer of air is reached, the value of n is unity. The extreme possible obliquity of the ray is 90°, when the sine is also unity. If the behavior of rays passing through the intital layers with increasing obliquity is studied it is found that light can emerge into the air layer provided that the constant product $n \sin i$ does not exceed unity; if it does so, however, the ray is found to be *totally reflected* and will not pass through the layer of air. However, if the air is replaced by a layer of immersion oil (refractive index about 1.515) the value of the above product can rise to this numerical value before the occurrence of total reflection.

The value of the refractive index of a medium increases with the frequency of the vibration in the light, and the bending of a ray having a given angle of incidence thus increases slightly with the change from the red towards the violet end of the spectrum. Since white light can usually be said to comprise all frequencies of visible light, the refraction of a ray of

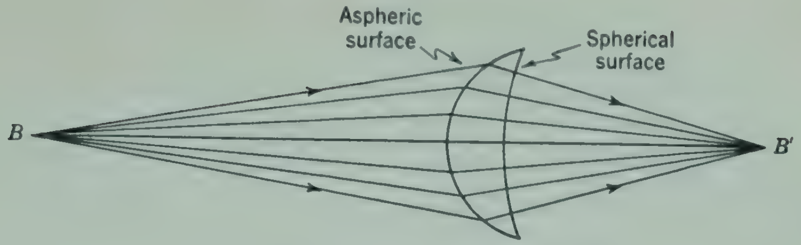


FIG. 4. Formation of a real image.

white light is accompanied by its *dispersion* into a narrow fan of rays of differing color.

3. LENSES. REAL AND VIRTUAL IMAGES

When a ray passes into a medium with a higher refractive index, the law of refraction indicates a value of i' smaller than i, and the ray is bent towards the normal; the bigger the angle of incidence the bigger the deviation. Refraction of monochromatic rays at a curved surface is shown in Fig. 4. If the surface is given an appropriate aspheric symmetrical shape depending on the refractive index, *all* the rays of a single frequency derived from one point B may be re-united in another "conjugate" point B′ in the second medium. However the region of the second medium may be terminated by a spherical surface concentric with B′ leaving a "lens-shaped" body which still produces the perfect ray-union.

The point B′ is a *'real'* image of B. If such an image is received on a screen and observed while the aperture of the lens is varied, the illumination of the image is roughly proportional to the area of the lens surface from which the rays are derived.

Consider now the refraction shown in Fig. 5, where the rays are derived from a point B in a medium of higher refractive index than the second medium, and the refracting surface is convex to the incident light. The rays are now bent away from the normal, and again, if the surface is given an appropriate symmetrical shape all the final ray directions may be made to pass through a single point B′.

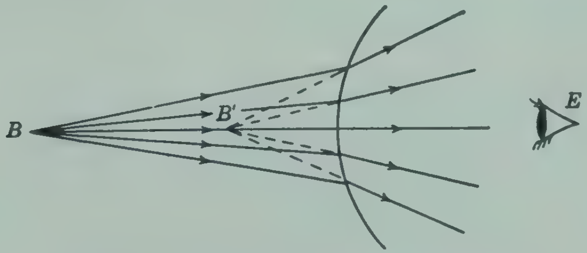


FIG. 5. Formation of a virtual image.

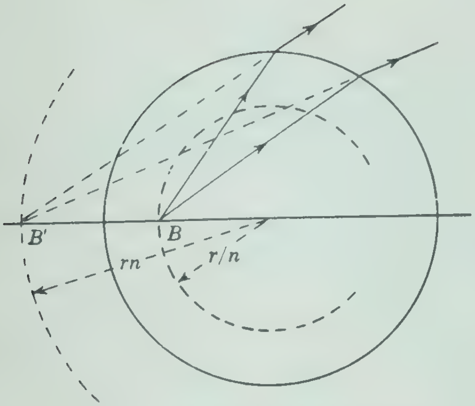


Fig. 6. Aplanatic refraction.

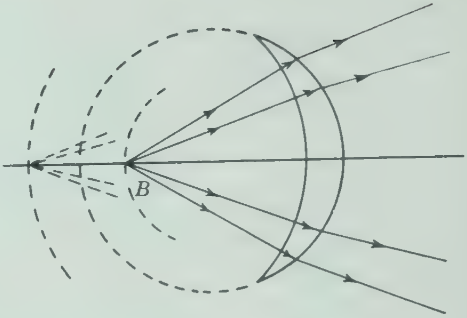
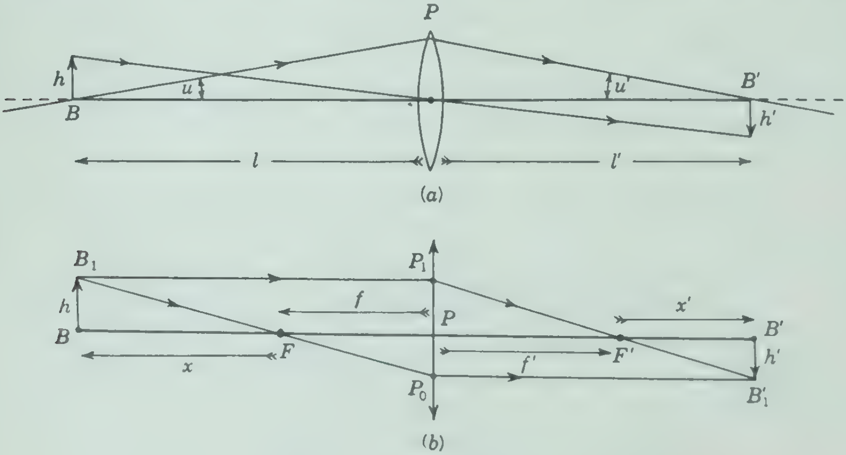


Fig. 7. Aplanatic lens.

The point B′ is now a "*virtual*" image of B; the image cannot be received on a screen, but it could be seen by an eye E receiving the refracted rays.

There is an important case in which the use of a *spherical* surface permits the formation of a perfect virtual image; see Fig. 6. Imagine a sphere of glass (in air) of refractive index n and radius r; imagine further two concentric spheres of radius r/n and rn respectively. Then rays from a point B on the inner sphere (radius r/n) will have a perfect image from the point B′ on the outer sphere, the points B and B′ both lying on the same radial line. It is the case of "*aplanatic*" refraction, and can be used in a *lens* if rays derived from a point in air, Fig. 7, are first refracted at a concentric spherical surface which leaves them unchanged in direction; they can then be refracted aplanatically. The lens of Fig. 7 produces a perfect virtual image by the use of spherical surfaces.

Figure 8 suggests the action of an equi-convex lens producing a real image with finite conjugate distances. In order to produce *perfect* ray re-



(a)

(b)

Fig. 8. Action of thin lens.

union one or both of its surfaces would have to be aspheric. However, if they are replaced by the spherical surfaces of closest fit and the aperture is kept relatively small, the ray union given by such a lens is still fairly satisfactory. Sharp photographs can be taken in monochromatic light by a double-convex lens with spherical surfaces provided the aperture is very small; but if the aperture is enlarged, the rays from an axial object point will not pass through a single image point. The defect is called *spherical aberration.*

If white light is used the dispersion of the medium will cause the blue rays to focus nearer the lens then the red. This is one form of *chromatic* aberration. Provided the aperture is small enough and the light is monochromatic, the lens will give a fairly sharp image, no matter what the distance of the axial object. It can be shown that the *deviation* of a ray passing through the lens at a small distance above the axis is practically constant for small changes of direction of the ray; thus the bent ray could be simulated by a piece of wire BPB′, Fig. 8(a), bent through a small angle at the pivot point P. The crossing points of the wire with the axis represent conjugate points.

4. Simple Conjugate-distance Relations

The slope (u) of the ray is negative if it rises from left to right, and vice versa; a measure of the initial slope is the height (y) of the ray in the lens (positive above the axis) divided by the conjugate distance (l); hence in accord with the sign of the slope, the conjugate distance will be called negative if the point lies to the left of the lens, and vice versa.

The slope and conjugate distance after refraction are distinguished by a prime, thus: u', l'.

The property of constant deviation of the ray is formally stated thus: Slope after refraction *minus* slope before refraction = (constant) deviation. Algebraically this is written:

$$\frac{y}{l'} - \frac{y}{l} = \text{constant.}$$

Further, it can be shown that the deviation itself is proportional to y, the intersection height; let it be yF say; then F is called the power of the lens, and

$$\frac{y}{l'} - \frac{y}{l} = yF, \qquad \text{or} \qquad \frac{1}{l'} - \frac{1}{l} = F. \tag{3}$$

This is the simplest form of conjugate distance relation; it lends itself to the discussion of some useful terms. Suppose for example, the object point is infinitely distant. Then $1/l = 0$. The resulting value of l' is then called the focal length, usually written f'; i.e. $1/f' = F$. Similarly we may find a value for l such that l' is infinite; this will clearly be numerically equal to the focal length though it will differ in sign; it is called f.

The point conjugate to an infinitely distant object is called the principal focal point (image space), or the "second principal focus"; conversely the point conjugate to an infinitely distant image is called the principal focal point (object space) or the "first principal focus".

It is easily shown (by a little algebra) from the above relation that if the conjugate distances are measured from the corresponding principal foci [Fig. 8(b)] instead of from the lens, being called x and x' respectively for the object and image distances, the conjugate-distance relation takes a new form i.e.,

$$xx' = ff' \tag{4}$$

This relation is known as Newton's equation. The sign convention is agreeable to that used above.

5. Magnification

Discussions so far have concerned only the axial conjugate points; it can be shown, however, that the property of reasonably good image-formation holds for points sufficiently near the axis in object and image *planes* intersecting the conjugate points and perpendicular to the axis; thus a point in an object-plane has a unique point in the conjugate plane. We can thus find the image-plane if we can trace two rays from the object point through the lens to their point of intersection. Consider the point B_1 in Fig. 8(b). The ray B_1P_1 parallel to the axis must be refracted through the second principal focus F'. Similarly the ray B_1F through the first principal focus must be parallel to the axis on emergence. These paths intersect in B_1', thus defining the focal plane and the value of h' ($B'B_1'$) corresponding to the object height BB_1. The dimensions in the figure are entered according to the definitions above. Then from the geometry of the figure, the triangles BB_1F and PP_0F are similar; since $PP_0 = h'$ we get for the linear *magnification* (h'/h)

$$\frac{h'}{h} = -\frac{f}{x} \tag{5}$$

the negative sign being necessary since h' is negative in the figure as well as f and x. Similarly it is easily seen that

$$\frac{h'}{h} = -\frac{x'}{f'} \tag{6}$$

Equating these alternative values of the magnification, we recover Newton's equation.

The magnification of the complete microscope is discussed in II, 1, below.

6. Properties of "Thick Lenses"

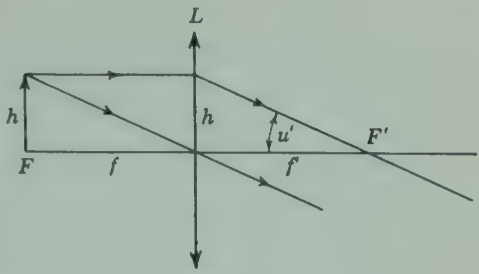


Fig. 9

Within certain limitations which need not be discussed here, thick lenses and lens systems such as objectives and eyepieces each possess a pair of principal focal points, and their magnification is found to obey relations like (5) and (6) above; so that, for example, the magnification of an objective is directly proportional to the distance of the image from the second principal focus. The *constant* of *proportionality* is then $-1/f'$ where f' is said to be the "focal length" of the lens, though it cannot be measured (as with a thin lens) by finding the distance between the surface of the lens and the principal focal point.

There is another analogy, which will appear from Fig. 9. An object of height h is situated in the first principal focal plane (through F) of a thin lens; its image is 'at infinity' and all the rays are parallel after refraction; the ray incident parallel to the axis is refracted through F′, and the angle u' of slope between the parallel rays and the axis is clearly represented by h/f'.

$$u' = \frac{h}{f'}$$

It follows that the focal length of the lens determines the angle under which its image will be seen when the object lies in the first principal focal plane, and is measured by h/u'.

This relation is also true for thick lenses or combinations of lenses like objectives and eyepieces (or indeed for a compound microscope) and it affords another method of measuring the focal length if necessary.

The combination of lenses to form a compound microscope is shown in Fig. 10. See II. 1.

There are important reasons why thin lenses of small aperture cannot be used as microscope objectives. As will be seen below, the wave nature of light calls for wide apertures; aspheric surfaces cannot readily be made; so that the use of complex systems in which the spherical aberration produced at once surface is annulled by others is essential. The effects of dispersion

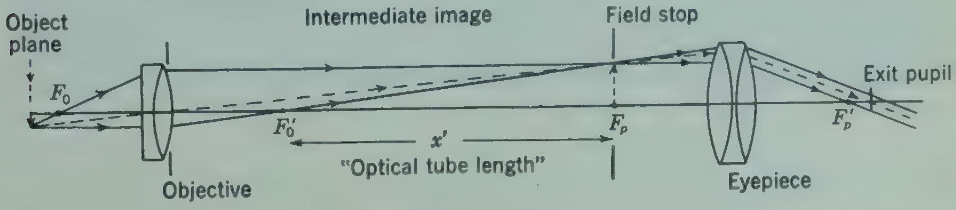


Fig. 10. Ray diagram for a compound microscope.

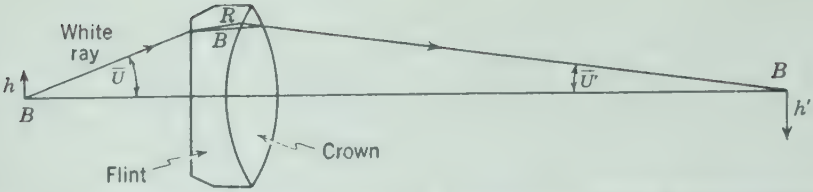


FIG. 11. Action of achromatic doublet. (Angular deviations exaggerated.)

also have to be annulled by the use of lenses including "diverging" lenses made of media of relatively high dispersion, e.g., flint glass.

The mode of action of an achromatic lens is suggested by Fig. 11, though the dispersion effects are exaggerated. The red and blue rays are indicated by R and B respectively. The whole action depends on the appropriate balance between the powers of the lenses and the dispersions of the glasses. If for example we use a flint lens of the same refractive index for the mean ray, but of a still higher dispersive power, we could make the final focus of the blue ray further away from the lens than the red; this would be called *over correction*, because the condition is the converse of the case of the uncorrected lens mentioned above.

The achromatic lens should also exhibit compensation of spherical aberration, the effect of the outer surfaces being more or less balanced by the inner one. If however the aperture is much increased the aberrations of opposite signs grow with the aperture at different rates so that no real balance is possible and more complicated systems must be used, as will be indicated below. If a perfect lens forms the image of a very small source of light the image, highly magnified, looks like Fig. 12(a); the effect of spherical aberration may be like Fig. 12(b).

7. The Optical Sine Relation and Sine Condition

When the use of a thick, properly corrected system enables sharp image formation to be achieved in the presence of rays making considerable angles (U, U') with the axis, there is an important relation between the sizes of

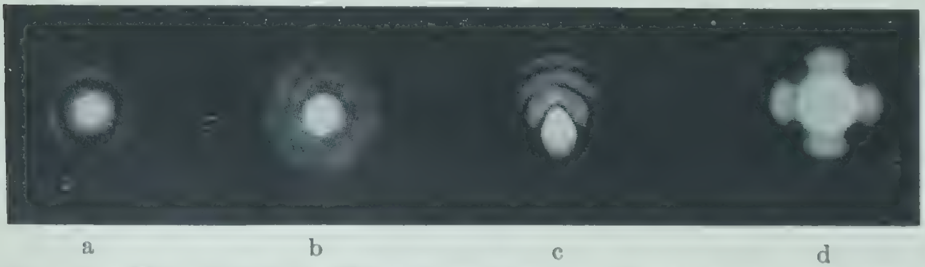


FIG. 12. "Star discs". (a) Well-corrected system. The Airy disc. (b) Effect of spherical aberration. (c) Effect of coma. (d) Effect of astigmatism. (Image midway between the two focal lines.)

object and image and the corresponding inclination of a ray passing through their axial points before and after refraction. It covers the case when the refractive indices of the object and image spaces may differ, and is thus stated:

$$nh \sin U' = n'h' \sin U'. \qquad (7)$$

From the sine relation it follows that in order to retain a constant value of h'/h for all zones of the system, the necessary condition is

$$\frac{\sin U'}{\sin U} = \text{constant.}$$

If this condition is not secured by the design of the lens system, the physical image of an off-axis object point is subject to a lack of symmetry around the principal ray, which, when very marked, takes the form of a comet-shaped flare extending inward or outward in a radial direction. This aberration is known as coma. A "star image" affected by coma is shown in Fig. 12(c).

8. Oblique Astigmatism and Curvature of Field

The optical image of an off-axis object point formed by a system truly symmetrical around an axis has symmetry with respect to a plane containing this axis and the object point, but even if coma is absent there may be "oblique astigmatism." As a focussing screen is moved towards the lens the image concentration may take the successive but merging froms of a short line radial with respect to the center of the field, a more or less circular patch, and a short line in a direction perpendicular to the first. If the object points lie in a field symmetrical about the axis, all the focal lines lie on two curved surfaces also symmetrical about the axis, which touch each other at the axial image point. The surface containing the radial lines is called the "sagittal" focal surface, and the other is the "tangential" focal surface. The "star image" midway between them may look like Fig. 12(d).

By suitable design of the lens these surfaces may sometimes be merged into each other with lessened curvature, but even so the resulting field of sharp focus may still be curved, as it generally is in high-power microscope lenses of the ordinary types. By "over correcting" the astigmatism, it may again appear as the surfaces separate, but this curvature will be decreased and a compromise in this respect results in a general improvement in the "flatness of the field."

II. The Eyes and Vision

1. The Requirements for Optical Systems in Relation to Visual Needs

From the optical point of view each eye constitutes a camera, in which the image formed by the optical system falls upon the sensitive surface of the retina. References should be made to appropriate text-books for a full anatomical description. Figure 13 shows only a very simplified diagram of the horizontal section of the right eye approximately; the positions of the two principal foci of the optical system are shown by F and F′. The principal foci move axially closer to the optical system when accomodation is exerted. When a small *sharp* image is formed upon the retina, its size h' is given by Eq. (5) above written in the form[1]

$$h' = -f_e\left(\frac{h}{x}\right)$$

where $-h/x$ is the angle subtended by the object at the front focal point F, and f_e is the constant of proportionality; it is the (numerically negative) object-space focal length of the eye, and changes from about 17 mm down to about 14.2 mm when the accommodation is so far exerted as to bring the "near point" of vision from infinity to a distance of just over 100 mm from the cornea; at the same time the point F moves inwards from about 15.7 mm to 12.4 mm in front of the cornea. The net result of these changes can, however, be expressed sufficiently nearly for present purposes by referring the angular subtense of the object to the "nodal point" (N) of the eye, which only changes its position by about half a millimeter on accommodation. This lies about 17 mm in front of the retina (thus $F'N \approx f_e$) and has the property that (approximately speaking) the sizes of the retinal images would correspond to those formed by a pinhole at N instead of the lens system; we can thus draw rays straight through N, and neglect its change of position for rough discussions.

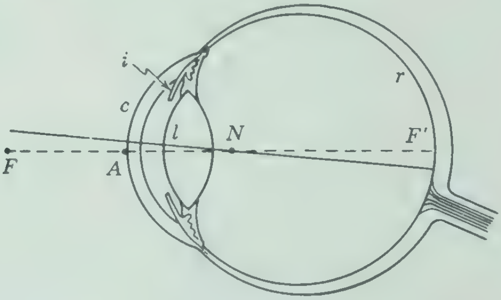


FIG. 13. Section of right eye (diagrammatic). i, iris. r, retina. c, cornea. l, lens.

When a small object of height h is viewed by the unaided eye (accommodated) it is held at the near point, at a distance b (say) from the nodal point, and the angle under which it is seen will be $-h/b$.

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[1] In accordance with a standard sign convention x will be numerically negative in the case shown in Fig. 8*b*. The beginner can disregard the signs; but they indicate whether images are erect or inverted.

When, however, a lens is used to 'magnify' the object, the latter can be held in the front focal plane of the lens as in Fig. 9, and the eye views the image at infinity; the angle under which the image is seen is (as above) h/f_p', where f_p' is the focal length of the magnifying lens.

Since under any conditions the apparent size of the thing or image seen depends upon its angular subtense at the nodal point we may write

$$\text{Magnification} = \frac{\text{Angle under which image is seen with optical aid}}{\text{Angle under which object is seen at 'near point'}} \approx -\frac{h/f_p'}{h/b} = -\frac{b}{f_p'} \qquad (8)$$

In order to compare the magnifying powers of various lenses, a conventional value is often given to b, i.e. -250 mm. Thus an eyepeice of 25 mm focal length would magnify about ten times when used in the above manner. If an eyepiece is used to magnify the image projected by an objective (see Fig. 10),

"Total angular magnification"

$$= \text{(Linear magnification of objective)} \times \text{(Angular magnification of eyepiece)}$$

Thus, combining Eq. (6) and (8),

$$\text{Total angular magnification} = \frac{x'}{f_0'} \cdot \frac{b}{f_p'}$$

where f_0' has been written for the focal length of the objective, and x' is the "optical tube length", i.e., the distance from the upper principal focus of the objective to its image plane. This may be about 160 mm in a modern instrument, so that a 4-mm objective would have a linear magnification of 40. Used with the eyepiece mentioned above, the total magnification would be 400.

Some makers adopt a different system of reckoning the magnification of objective and eyepiece, but the final result is the same.

The magnification required to "see" a fine structure depends in the first instance on the retinal grain. The sensitive elements of the retina are most closely grouped near the *fovea centralis*, where the centers of the so-called cones are separated from each other by about 2×10^{-3} mm; this interval subtends an angle of about half a minute of arc at the nodal point. It has long been known that the limit of perception for an object such as a double star is somewhere near one minute (0.00029 radians), but details separated only by one minute seem very "tiny". (Imagine, for example, a millimeter scale at a distance of 3½ meters.) The magnification for images of good contrast must therefore be adequate to bring up the angular subtense of the finest intervals to at least four or five times this value, say 0.0012 radians.

Take for example a diatom of which the frustule shows a regular pattern, for example the well known *Amphipleura pellucida*, which may exhibit a ribbed structure with an interval as small as 0.25×10^{-3} mm. Viewed at 250 mm, the angular subtense of the interval would be 10^{-6} radian. Hence in this case (writing m for the magnification) $m \times 10^{-6} = 0.0012$, and $m = 1200$. More generally for high-contrast detail,

$$m \frac{h}{250} \approx 0.0012,$$

or

$$m \approx \frac{0.3}{h},$$

where h is the size of the finest object detail in millimeters.

The limit of 1 minute for perception of fine detail is only true for objects of high contrast, suitably and adequately illuminated; if the contrast of light and shade is weak, or if the perception of detail has to depend on the detection of color differences as in some "stained" preparations, *much* larger angles may be required and the magnifying power will have to be increased accordingly, especially if easy perception is essential. The whole subject has been investigated by Arnulf (1937) and his collaborators. It has long been known that the limit of angular perception does not improve much with the luminance or brightness of the field provided that this is above about 10^{-5} candles per square centimeter, or *comfortably bright;* the use of over-bright fields should be avoided in the microscope because the field of the instrument occupies only a fraction of the whole possible field of vision, and the eye does *not* function so well in general unless the whole retina is adapted to the light. In visual observations, the provision of a *wide field* makes for greater comfort and more efficient seeing. Arnulf studied (*inter alia*) the limit of separation for objects ("gratings") of fixed brightness but varying contrast, and found that with very weak contrast the limiting angle may easily rise by a factor of 20 or 30. Hence the numerical discussions above can only be regarded as a very rough guide applicable in some cases of high contrast images.

Naturally the choice of magnification for a picture has to depend on other considerations beside that of exhibiting the smallest detail present. In many cases the limit of perception is set by the available objective (see below) and it is useless to employ a magnification higher than that appropriate to the instrumental limitation. In other cases it may be of importance to show the whole of a given region of the object in one picture regardless of resolution.

There are important reasons *against* the use of very great magnification, especially by the use of very high-power eyepieces. The light enters the eye in a beam which is restricted to a very narrow diameter (in the "exit pupil"[2] of the microscope) near the region where it crosses the eye pupil. Owing to the optical imperfections and irregularities of the eye, the presence of local opacities in the "vitreous" and the like, there may be troublesome shadows looking like wavy filaments, etc., which interfere to some extent with the ease of vision. They become prominent and objectionable as the size of the exit pupil decreases, as it does with increasing magnification. It will be shown below that more and more detail may appear with increase of magnification until the exit pupil shrinks to a diameter of about half a millimeter; but these "entoptic" phenomena are sufficiently troublesome to discourage any further increase. They affect some eyes more than others.

2. Effects of Ametropia

The human eye may suffer from various "refractive" errors. The defects of ordinary hyperopia, myopia, etc. are easily corrected by adjustment of the focus when using a microscope; some eyes are, however, very astigmatic, and, while this cannot be corrected by the ordinary instrument, it can be countered by the use of a small appropriate "cylindrical" lens appropriately mounted over the eye lens of the instrument. Spectacles can be worn if the eyepiece is such as to give a sufficient clearance to the exit pupil. Care should be taken to ensure that the cylindrical correction is tested with a very small aperture in front of the eye as it may differ perceptibly in direction and amount from the value found when the whole of the eye-pupil is operative. Corrections of less than half a diopter of astigmatism do not usually give appreciable trouble if omitted.

Prolonged use of the microscope requires a comfortably inclined position of the head and relaxed visual accommodation. Beginners are apt to "screw up" one eye and exert considerable accommodation with the other when using a monocular instrument, a condition which results in strain and discomfort. The provision of a black mask, Fig. 14, cut from cardboard and slipped over the end of the tube allows the instrument to be used with both eyes open, the unwanted field of vision being occluded. The observer should try to look "through the bench" when focussing.

3. Binocular Vision and Its Requirements

Human vision may be considerably more satisfactory when both eyes are employed rather than one alone, especially if there are slight residual

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[2] The "exit pupil" is the image of the effective objective aperture formed by the eyepiece and is seen behind the eyepiece when the objective is fully illuminated. (See fig. 10.)

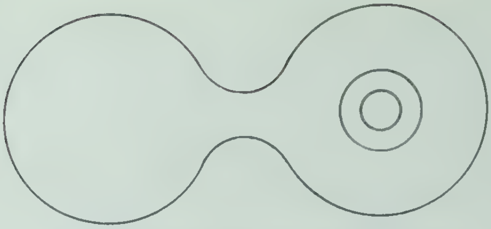

FIG. 14. Mask to screen left eye.

defects in monocular vision. This applies even to the vision of fine detail in a plane where no question of "relief" arises. The "entoptic" phenomena etc. of the one visual field tend to be suppressed by the other. To borrow a simile from the field of "radio" the "signal to noise" ratio is much improved. However, the use of both eyes in the ordinary way on objects at distances of less than a few yards results in the sensation of "stereoscopic relief" which is caused because each retinal image can be looked upon as a special perspective projection with regard to the corresponding nodal point as center; the separation of the centers thus produces slightly different pictures which are "fused" in the binocular visual images and produce the experience of "relief."

There is much to be said for the use of a properly adjusted binocular microscope when prolonged and exacting observations have to be made, though some observers find it difficult to maintain the comfortable balance between accommodation and convergence which is automatic in ordinary vision; the lack of such a balance when using an instrument may cause difficulty unless the adjustments are very carefully made. When an object can receive prolonged and leisurely study it is probable that little extra can be seen with a binocular instrument not finally visible in the monocular one.

As regards stereoscopy, it can be produced in the high-power microscope only at the expense of some loss of numerical aperture and consequent risk of impairment of the image. The spatial relief of an object can be studied with a monocular instrument by adjusting the focus quantitatively so as to bring successive "planes" into focus, and this without any diminition of aperture. Practically all modern binocular instruments can be made to give stereoscopic relief by simple means if required, and in high-power work it is not generally claimed that stereoscopic observations offer any great advantages. On the other hand low-power binocular microscopes with independent objectives, frequently used for magnifications between about 10 and 100, employ stereoscopic vision to very good effect, especially when used in conjunction with micro-manipulators (Barer and Saunders-Singer, 1951) and dissectors where instantaneous appreciation of the three-dimensional structure of an object is essential.

Binocular Microscope for research. Cooke, Troughton, & Simms, (York).

III. The Wave Nature of Light and Its Consequences

1. Interference and Diffraction

Consider a microscope objective typified by Fig. 11, which is forming the image B′ of a very fine pinhole in a silver film (suitably illuminated) so that for practical purposes it resembles a "point source" of light. The rays from

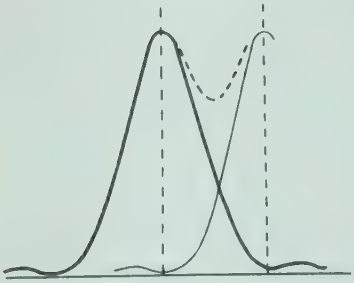

FIG. 16. Airy-disc intensity (solid line), and summation of intensities for over-lapping discs (broken line).

B may have some residual defects in their re-union near B′; the image has an appreciable size. If these "aberrations" are bad, the reduction of the aperture of the lens by a symmetrical diaphragm cuts out the outer rays which tend to be "wide of the mark," and the image size decreases; but (owing to the wave nature of light) if the aperture is still further reduced the diameter of the patch of light tends finally to increase and to be inversely proportional to the aperture. The image now seen (under sufficient magnification) is suggested by Fig. 12a (see also Fig. 16) and the phenomenon is known as the Airy Disc. Even if the lens is so perfectly corrected that *all* the rays pass through B′, this appearance will still be seen, and the radius ρ of the dark ring surrounding the central concentration will be given by

$$\rho = \frac{0.61\lambda}{n' \sin U'}, \tag{9}$$

where λ is the wave length of light, and U' is the angle between the axis and the extreme ray in the symmetrical bundle passing through B′. The value of the refractive index n' is, of course unity if the medium is air.

This phenomenon is (as mentioned above) explicable in terms of the wave nature of light. Waves diverge from the source, just as ripples spread outwards from the point where a pebble is dropped into a pond. The function of the lens is to catch some of the "ripples" and make them convergent instead of divergent, so that they tend to bring their energy into the region of the focus. Huygens suggested, in 1690, a pictorial way of discussing the action. According to him, each point on a wave can act as the origin of new wavelets, and it is the action of all such wavelets at any point which produces the effects observed. If this principle is logically followed for the case of rythmic vibrations of a single frequency the occurrence of the Airy Disc can be deduced, and also its finite size in three dimensions; it persists for a certain depth of focus on both sides of the best focal plane.

Imagine now that a lens L, Fig. 17, produces plane waves of light, and we obstruct the wave by a screen containing two very small apertures. The rythmic pulses enter the apertures and (just as in Huygens' principle) we

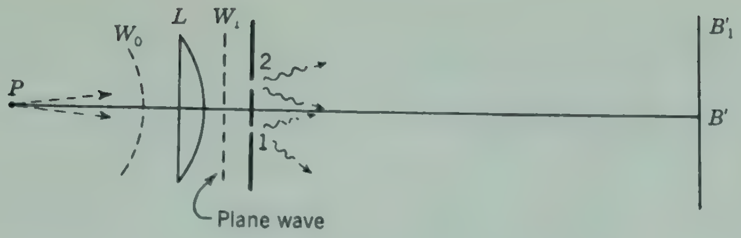

FIG. 17. Production of interference fringes.

now have wavelets spreading outwards from these places, which can be pictured as tiny radio stations. Let us suppose that the vibrating effect of either station at some point in its field of influence can be described as a rythmic succession of "lifts" and "depressions" in the medium, just as the water wavelets are constituted by lifts and depressions of the surface. A symmetrically-placed point B′ equidistant from the station receives pulses from each with exactly the same timing, so that the signal strength is doubled. However, consider a point B_1' which is rather further away from aperture (1) than from (2). Since the waves from (1) take longer on the journey we shall find a point (as we move away from B′) where instead of getting 'lift and lift' in exact coincidence, a lift from (1) will accompany a depression from (2), and vice versa, so that in this region the effects will tend to annul each other. The distribution of "amplitude" in the plane B_1' is found in fact to show a regular variation as suggested in Fig. 18 curve b. The appearance, which can be photographed or viewed with a magnifier,

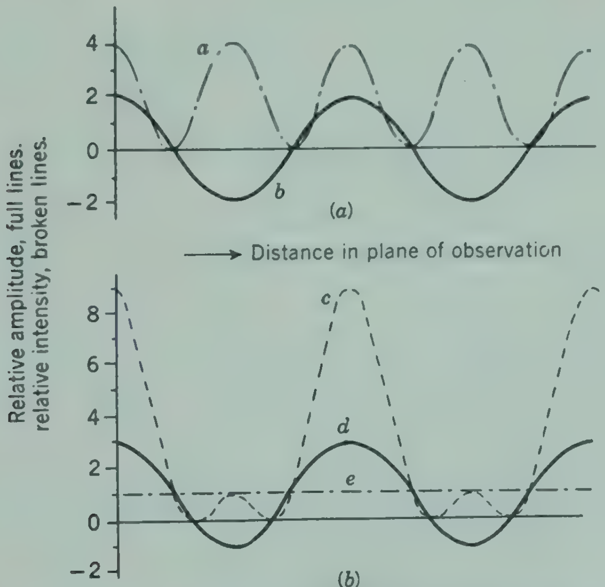

FIG. 18. (a) Amplitude and intensity curves, two apertures. (b) Amplitude and intensity curves, three apertures.

shows alternating light and dark regions called "interference fringes"; Fig. 18 curve a, shows the intensity variation, which is proportional to the square of the amplitude. Regions in which there is no displacement separate regions of heightened vibration, but it will be found that if P, Q, R, S, T, etc., are successive dark fringes, the momentary displacement between Q and R is always opposite in sign from that between P and Q or R and S.

Now suppose that a third aperture of equal size is opened up between apertures 1 and 2. (We suppose that the central point B′ is so far from the screen that there is no significant difference between *its*' distance from any of these apertures). Then the middle aperture contributes a vibration of unit amplitude to the whole interference field (see fig. 18, curve d); the maximum displacement in the center of the field is raised to +3, while the relative displacement on the other side of the dark fringe is diminished to −1. The action of three slits is represented by plotting the square of the ordinate in the summation curve, Fig. 18, curve c. The main maxima in curve c are at twice the distance of those in curve a, and they are separated by secondary maxima of much smaller amount. Note that the spacing of the three slits is half that of the first case.

The main lesson to be grasped from such arguments is that the mutual action of a few equidistant sources of "coherent" vibrations can produce a wide variety of interference effects according to the amplitude and phases (or relative timing) of the contributions.

Arguments of this kind used above can be continued; if for example we have five equidistant slits the main maxima are found to be separated by three secondary maxima of relatively small amount. In fact if there are a great number of slits, the secondary maxima become negligible and all the visible light will be concentrated into the main diffraction maxima. In order to see these properly when there are a great number of slits the diffracted light from the slits is collected by a lens which brings the vibrations together without introducing any further path differences. The direction of the "diffracted wave" W′ Fig. 19(b) is such that the distance beween it and the successive apertures increases by a complete wave length of light (λ) at each step; thus the timings of the successive pulses support each other.

There are other diffraction maxima for the directions in which the distance between the diffracted waves and the successive apertures increase by 2λ, 3λ, etc; see Fig. 19(a); moreover if the incident wave is symmetrical there will be symmetrically diffracted waves on the other side of the normal.

The occurrence of diffraction in this way is easily observed when a small source of light is observed through a screen with regular and finely-spaced apertures; for example a distant street lamp seen at night through an umbrella. The lens of Fig. 20 is then represented by the lens of the eye, and the various diffraction maxima are formed on the retina.

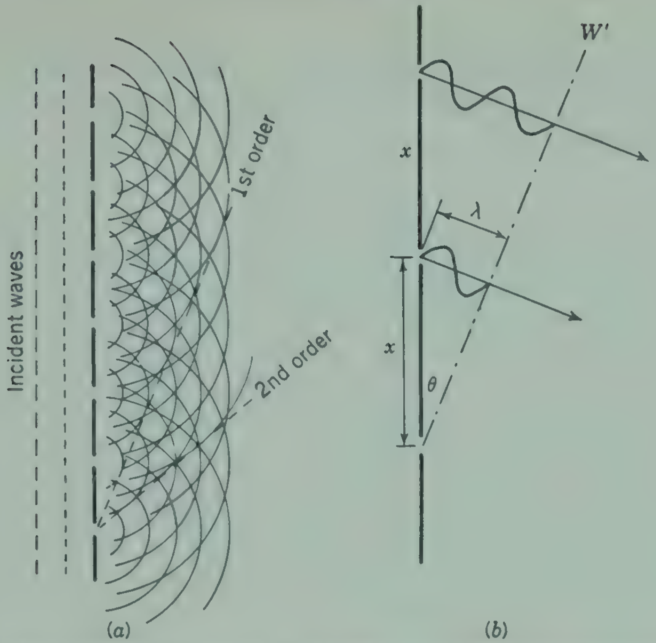
Incident waves
1st order
2nd order
W'
x
λ
x
θ
(a)
(b)

FIG. 19. Diffraction by a grating. (a) The diffracted waves on one side of the normal are seen as the envelopes of wave fronts spreading from successive apertures. (b) The exact direction of the first-order wave is seen as related to the wave length and aperture-interval.

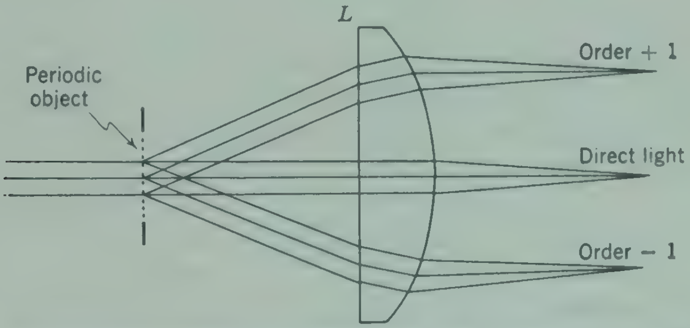
L
Periodic object
Order + 1
Direct light
Order − 1

FIG. 20. Collection of diffracted beams by the microscope objective. (The incident light is normal.)

Now if x is the interval between the apertures and θ the angle of diffraction, the first condition discussed above (see Fig. 19(b)) is given by

$$x \sin \theta = \lambda \tag{10}$$

For the other diffraction maxima, their directions are given by

$$x \sin \theta = p\,\lambda \tag{11}$$

where p may have integral values either positive or negative. If p is zero, it corresponds to the wave which travels onward in the original direction. If the medium in which the diffraction takes place has refractive index of n, the wave length corresponding to a given frequency will be λ/n instead of the λ in air, so that (10) becomes

$$x \sin \theta = \lambda/n \tag{12}$$

Referring again to Fig. 20, let us regard the lens as the objective of a microscope. When the object is illuminated by a single parallel beam, the light entering the objective consists of the direct beam *plus* various diffracted beams. In the case of fig. 20, the objective brings all the light into three well-defined maxima in its upper focal surface. The light spreading from these maxima will produce "interference" effects in the image plane conjugate to the object in a way very similar to that discussed in connection with Fig. 18(b). It can be shown that these interference effects constitute an 'image' of the object. See the textbook by the author (Martin, 1950).

The relevance of the above discussion to the action of the microscope has, of course, still to be explained.

2. RESOLUTION AND THE REQUIREMENTS OF OPTICAL SYSTEMS IN RELATION THERETO. NUMERICAL APERTURE

In commencing the study of image formation in the microscope, it is convenient to begin with an imaginary 'object' consisting of an equispaced row of infinitesimal pinholes in an opaque plane. The optical system can be supposed free from aberration. Each pinhole is imaged as an 'Airy disc', and the central patches almost "touch" when the first dark ring of one falls on the corresponding ring of its neighbor. The condition is thus that h' is equal to twice the value of ρ given in Eq. (9) above i.e.,

$$h' = \frac{1.22\lambda}{n' \sin U'};$$

combining this with the optical sine relation (7) we get

$$nh \sin U = 1.22\lambda,$$

or

$$h = \frac{1.22\lambda}{n \sin U};$$

if the point-objects are any closer than this, the patches will not be seen as definitely separated by a dark interval, but they will still be quite *distinctively* recognized if they are a little closer, and

$$h = \frac{\lambda}{n \sin U}. \tag{13}$$

Let us look at this expression and compare it with (12) which (rewritten) gives

$$x = \frac{\lambda}{n \sin \theta}$$

The last equation tells us that if the limiting angle of the rays which can enter the objective is roughly the same as the angle of diffraction of a light-wave from the regular structure, then x (the mutual distance of the regular elements) is just about the same as that of two point objects which are distinctively separated in the image.

The product $n \sin U$, i.e., the refractive index of the object medium multiplied by the sine of the limiting angle (with the axis) of a ray which can enter the objective from the axial object point, is determined by the design of the lens, and is called the NUMERICAL APERTURE; symbolized by NA.

The above condition (13) does not represent the closest approach of the two apertures consistent with the inference (from the appearance of the image) that there were two instead of one. If the first dark ring of one falls on the maximum of the next (Fig. 16) there is still a slight drop in brightness between two maxima; the resolving limit L is usually written

$$L = \frac{0.61\lambda}{NA}. \tag{14}$$

This formula applies strictly to the case when the two apertures are independently illuminated; if the *row* of holes is illuminated, as in Fig. 20, by waves from once source (coherent light) the resolution would fail for a spacing as fine as that of (14); the diffracted light from any small region of the source would fall outside the aperture of the objective.

We might, however, still obtain resolution by the device of illumination by *oblique* coherent light, Figs. 21 and 22. If the incident wave is inclined at an angle θ and the diffracted one by an angle θ' the condition for a diffracted wave will be (allowing for a change of refractive index from n to n'

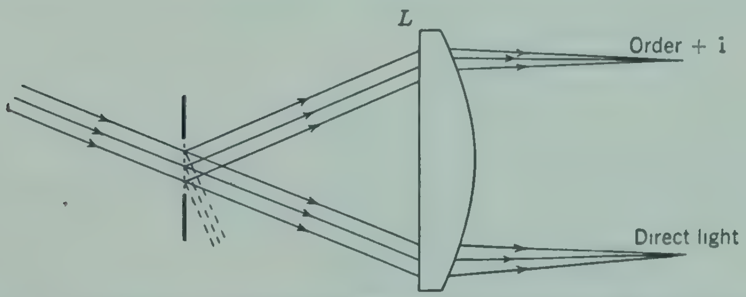

FIG. 21. Collection of diffracted beams. (The incident light is oblique.)

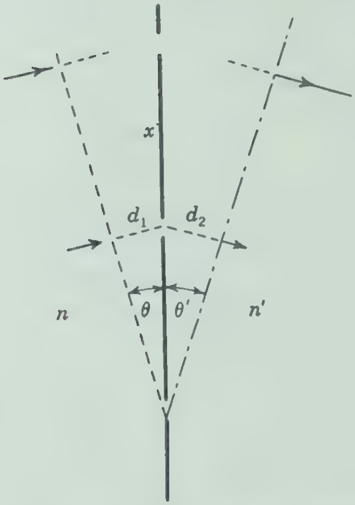

FIG. 22. Derivation of formula for the relation between the incident and difracted waves. The sum of d_1 and d_2 is equivalent to one wave length for the first order.

at the object plane)

$$nx \sin \theta + n'x \sin \theta' = p\lambda,$$

where p will again have integral values. For the zero order (or direct light, $p = 0$), and the angle of diffraction is θ_0'

$$nx \sin \theta + n'x \sin \theta_0' = 0$$

or

$$n \sin \theta = -n' \sin \theta_0'$$

This is simply the law of refraction. However if $p = 1$ the angle of diffracted light will have the opposite sign, Fig. 21, and

$$x = \frac{\lambda}{n \sin \theta + n' \sin \theta_1'}$$

The most advantageous condition would be that θ_1' should be equal to $-\theta_0'$. In this case we should have

$$x = \frac{\lambda}{2n \sin \theta},$$

and if θ corresponded to the maximum angle (U) accepted by the objective,

$$x = \frac{\lambda}{2NA},$$

which is not far from the limit expressed by (14). It may be inferred that if the light is "coherent" the illuminating light must be oblique or at least contain oblique rays of numerical aperture approaching that of the objective if the resolution is to be equivalent to that obtained by non-coherent light.

The term "resolution" as used in the above sense means that we obtain in the image a geometrical variation of brightness corresponding to the variation of the object structure. It enables us to infer one thing only, viz. the magnitude of the regular object-intervals.

Suppose that the object consisted of a "grating" of opaque "bars" and "spaces"; then it can be resolved in the foregoing sense if we can see some kind of regular brightness variation in the image with an interval depending on the magnification of the objective; but we cannot infer what is the ratio of the width of a "bar" to that of a "space" in the object. If there is simply such a periodicity in the image the grating itself might not have opaque bars and spaces at all, but might simply have a regular variation of transmission, as would be given for example by a transmission t corresponding to a linear displacement d where

$$t = A\left\{1 - \sin 2\pi\left(\frac{d}{x}\right)\right\},$$

A being a constant. This can be called a sine grating. How then can we ever infer what kind of object is really present? In order to discuss this question it is necessary to cite a principle due to *Fourier*, who showed that no matter what is the law of a regular transmission function, it can be represented by adding together a succession of "harmonic" functions (i.e., depending on sines and cosines). For example the square-topped function of Fig. 23, has a value of $\pi/2$ between $y = 0$ and $y = \pi$, changing suddenly to zero between $y = \pi$ and $y = 2\pi$, and so on. It can be represented by a set of sine terms as follows:

$$S = \pi/4 + \sin y + \tfrac{1}{3}\sin 3y + \tfrac{1}{5}\sin 5y + \text{etc.}$$

The curves in Fig. 24 show only the addition of the first two periodic terms, but already there is an approach to an imitation of the square top; and

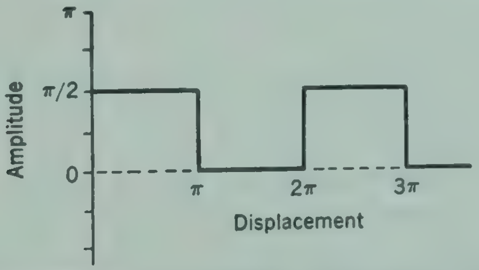

FIG. 23. Amplitude function for "grating" with equal "bars" and "spaces."

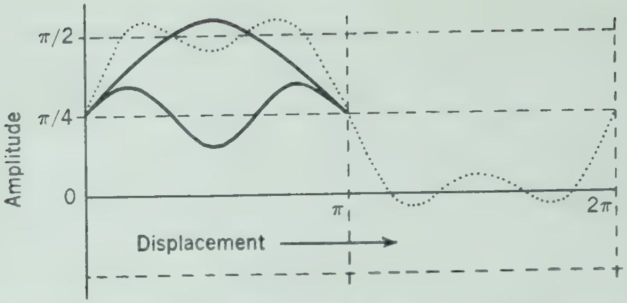

Fig. 24. Representation of function of Fig. 23, where the microscope can transmit only the first and second order maxima in addition to the "direct light."

the imitation gets steadily better according to the number of higher harmonics which can be added in.

In order to understand the way in which the microscope tries to form the image of a grating of equal bars and spaces, we can think of it as replaced by the sum of a set of sine gratings with transmissions and periods represented by the above series. Each grating produces its own diffraction maxima but the sine of the angle of diffraction is proportional to the grating frequency; so that the aperture of the objective would only be able to admit the maxima corresponding to a *limited number of the terms*; some of the diffracted light cannot enter the objective; the representation is thus more or less deficient; and the *difference between the required image and the actual one is represented by the missing terms.* The sum of such missing terms is itself periodic, so that instead of a sharply defined 'edge of a bar' we tend to get periodic variations of brightness or "secondary diffraction fringes." They do not represent any structure in the object itself.

If the object is a very coarse one the diffraction maxima tend to have smaller diffraction angles, so that a great number of the periodic terms can be represented in the image, and it looks correspondingly truthful; but when we have a structure "on the limit of resolution" only the first harmonic terms may be included and we can tell nothing about the "relative spacing of bars and spaces" but only that there is a "periodic" variation of transmission with a certain linear interval, in the object.

The above discussions have been largely based on the assumption that the object was illuminated by plane waves. We saw that the use of oblique light would permit the admission of direct and diffracted light when the "normal" illumination would fail, and it will therefore be understood that the use of a "condenser" which brings oblique waves as well as normal waves through the object offers advantages in securing resolution. Moreover the secondary diffraction fringes differ somewhat for the oblique rays and their total effects tend to annul each other, making a fuzziness at the "edge" rather than well-defined fringes. For such reasons it is desirable to increase the aperture of the condenser as far as possible towards that of the objective. The reasons limiting this increase will be discussed below.

3. The Abbe and Equivalence Principles

The essential parts of the above discussion of resolution were propounded by Abbe (1873) who showed that if a structure was to be "resolved" by the microscope, the direct light and at least the first-order diffracted light must be transmitted by the objective. He was the first to lay down the basis of the theory of image formation by coherent light.

When the condenser of the microscope forms (in the object plane) the image of a source of light, (so called "critical illumination") the condition is much closer to the case when the object elements could be said to be self-luminous. In fact when the numerical aperture of the condenser is equal to that of the objective the image formation is equivalent for most practical purposes to the self-luminous case. However, there are very few cases in practical microscopy when the acceptable aperture of the condenser is not considerably smaller than that of the objective. The theory of the image formation then turns on the degree of coherence (Hopkins, 1951) of the light. The present writer (Martin, 1931) and other authors have shown that there is *nothing critical* about "critical illumination." The use and function of the condenser is discussed below.

If undue prominence seems to be given to "periodic" objects like gratings, it is mainly because the theoretical discussion of such cases presents much less difficulty than most others. They serve, however, to introduce the more important theoretical principles. Other types of object are discussed below in Section VI.

The development of the theory of the microscope owes much to various authors other than those already mentioned, including Berek (1926), Conrady (1904), and Zernike (1942).

4. Depth of Focus

When a microscope objective, free from aberration and bounded by a circular aperture, forms the image of a bright point-object of negligible dimensions the image takes the appearance of the Airy disc; moreover as mentioned above the characteristic central concentration of the disc will persist for a certain range of positions of the image plane. Conversely it follows that there will be a certain tolerance of the position of the object plane for which this "concentration" is reasonably well marked in the one image plane. This "depth of focus" can be shown to be proportional to the wave length of light, and it depends also on the maximum possible angle (U) of obliquity of the rays passing through the object and entering the objective. There is thus a direct relation between the depth of focus and the numerical aperture of an objective. Given a mean wave length of light (0.5×10^{-3} mm) the correspondence is approximately as follows:

NA	Depth of focus in air (mm)	Depth of focus in medium $n = 1.50$
0.25	0.0079	
0.50	0.0019	
0.75	0.0008	
1.00		0.0007
1.25		0.0004

Many microscopic objects, living cells, thin sections, etc., have a finite thickness; the image seen in one focal plane B′ therefore consists of more or less sharp images of any sharply defined structure within such a range of focal positions as above, together with more or less diffuse images of the structures outside this range. Changing the focal position brings various planes of the object within the range of sharp focus. If the fine adjustment of the instrument is suitably calibrated the extension of the object in the axial direction can be measured with a possible error corresponding roughly to the depth of focus.

5. Systems for Illumination

The discussions of the foregoing sections will have emphasized the importance of high numerical aperture in securing efficient resolution. In a few cases (fluorescent objects, etc.) the object may be self-luminous; in other cases opaque objects may be illuminated from above (ring-lamp illuminators or reflectors) and diffuse their light over an adequate angle. However in the majority of cases of biological objects they are contained in a more or less transparent lamina between slide and cover-glass, and are illuminated by transmitted light.

The function of the condenser system is generally to illuminate the required area of the object as evenly as possible with the aid of the given source of light, and to supply light passing through the object with the required range of angular directions so as to be able, if necessary, to fill the numerical aperture of the objective (or at least to approach this condition). It is important that the total amount of light passed through the object shall not be greatly in excess of that needed to produce the image, since various internal reflections give rise to stray light and weaken the contrast in the image.

The design of condenser lenses is briefly described in Section IV, 4, below. They are usually designed so that when the source is at an equivalent

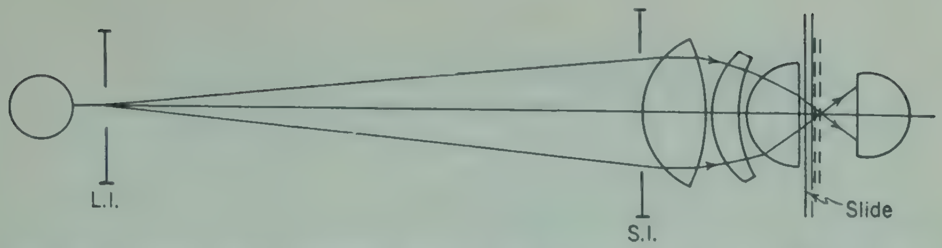

FIG. 25. Action of illuminating system (diagrammatic only). An aplanatic substage condenser is shown. LI, lamp iris; SI, sub-stage iris.

distance of about ten inches, its' image will be formed at a distance above the condenser lens surface adequate to allow the interposition of a slide of any reasonable thickness; a focussing motion then allows the image of the source to be focused in the plane of the object; Fig. 25.

The numerical aperture of the illuminating rays is now controlled by the substage iris diaphragm (SI). If the source of light is extended and uniform in brightness, such as an electric lamp with an opal bulb, the area of this to be employed can be controlled by the lamp iris (LI). The flat flame of an old-fashioned paraffin lamp made a useful source. If a lamp with an opal bulb is not available, white diffusing screens can be employed. A "pearl" or frosted bulb does not generally diffuse well enough to ensure even illumination.

When very low-power objectives are used, the area of the object plane thus illuminated may not be sufficient to fill the field of the microscope. In this case, it is sometimes sufficient to dispense with the substage condenser, and to image the source in the object plane with the concave substage mirror; though owing to the astigmatism associated with the oblique reflection some control of the light is lost.

There may be cases, however, in which the use of a source of greater intrinsic brightness or luminance is desired, to shorten the exposure in photomicrography. Such sources may be "point o'lite" or ordinary arc lamps, ribbon-filament lamps and so on; they may be so small that their image projected by the ordinary substage condenser lens (via the *plane mirror*) does not fill the field of the instrument with light or give uniform illumination. No attempt should be made to improve matters by the use of the concave mirror in such a case. The appropriate step is to use an auxiliary condenser A, Fig. 26, which is set up at an equivalent distance of perhaps 10 inches from the substage iris. The position of the source is now adjusted so that its enlarged image is projected into the plane of the substage iris, which it should fill when this iris is yielding the required condenser NA. The condenser C is now focused so that the iris fitted to the auxiliary condenser is imaged in the plane of the object. In this way control is established both of the aperture and area of the illumination. If the

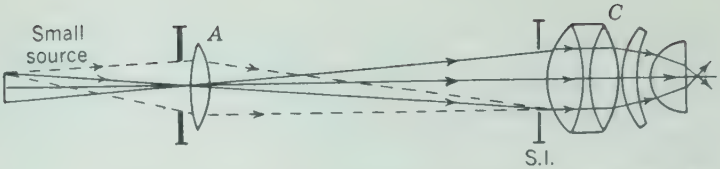

FIG. 26. Action of illuminating system with auxiliary condenser (A). An achromatic sub-stage condenser (C) is shown.

enlarged image of the source will not fill the substage iris, or if the image is irregular (as with a coiled filament lamp used as source) a ground glass or other lightly diffusing screen is used near the auxiliary condenser, so that the substage iris is evenly illuminated.

Such principles are employed in the design of illuminating systems "built in" to certain microscopes.

IV. The Optical and Mechanical Systems of Microscopes

1. Simple Microscopes and Magnifiers

Though the pioneers in microscopy such as Leeuwenhoek and Hooke successfully used simple microscopes with exceedingly small lenses of very short focal length, the difficulty of securing adequate numerical aperture with such lenses placed a limit on progress: though indeed this was only realized much later. The modern use of the simple microscope is mainly with low powers, for dissection etc., for example ×5 or ×10.

When an uncorrected double-convex lens is used as a low-power magnifier, Fig. 27, the chief noticeable trouble is the bad definition towards the outer parts of the field; if the eye is so placed that a ray from B_1 originally parallel to the axis is refracted through its nodal point, the image would be more satisfactory if a similar ray from B_2 were similarly refracted; however the spherical aberration of the lens causes the outer ray to be refracted too steeply thus producing *distortion* (pincushion type), and the chromatic aberration of the rays causes the image to show lateral color, the magnification of the blue image being greater than that of the red. The definition of the outer parts of the field is also affected by astigmatism.

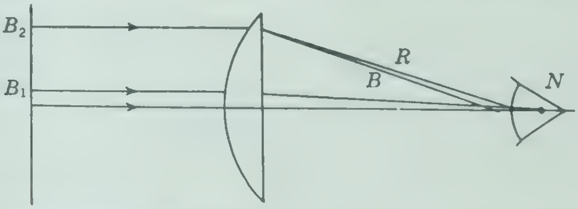

FIG. 27. Action of simple lens used as a magnifier. Origin of lateral color and distortion.

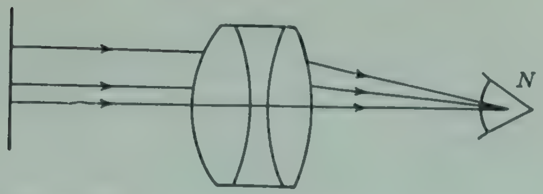

FIG. 28. Action of triplet-lens magnifier.

The image can be much improved by the use of an achromatic lens doublet or triplet Fig. 28 which also minimizes the distortion in the above sense.

2. OBJECTIVES AND EYEPIECES

The function of the objective, as discussed in sections I and III above, is to project an image of the object under some required magnification, making use of a cone of rays with the maximum possible aperture. The simple doublet typified by Fig. 11 develops serious aberrations if its *NA* is increased much above 0.1, and the "refraction" is better shared by two achromatic lenses, as in the type of objective due to Lister, for a range of *NA* from about 0.15 to 0.30, Fig. 29 (A). For still higher numerical apertures (0.65 or even up to 0.85) the device due to Amici of using a near-hemispherical front lens, Fig. 29 (B), is generally adapted. The curved surface can work near the condition of aplanatic refraction, but heavy spherical aberration can arise at the plane front surface, which is only minimized by bringing it as close to the object as possible. Space must, of

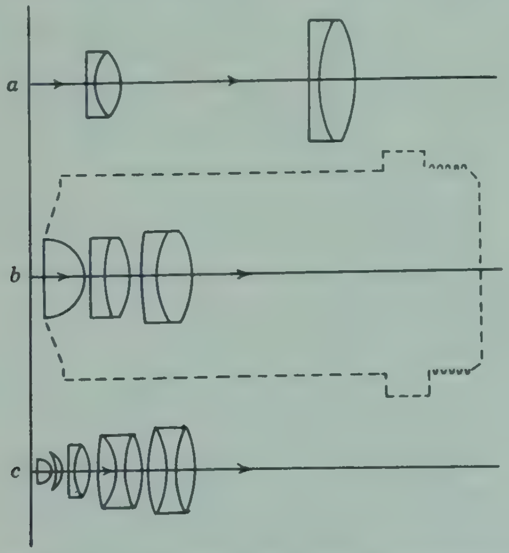

FIG. 29. (a) Lister-type objective. (b) Amici-type objective. (c) Immersion objective.

course, be left for reasonable variations in the thickness of cover-glasses, and there must be a reasonable allowance for the focussing, but the clearance is necessarily short and the more so the higher the aperture. The spherical and chromatic under correction of the front lens are compensated by over-correction of the back lenses. The occurrence of heavy aberration at plane air-glass surfaces thus sets a limit to the *NA* attainable with "dry" lenses and a reasonable working distance, but a great extension of aperture is possible in "immersion lenses," Fig. 29 (C) by filling the space between the lens and object by media of a refractive index comparable with that of the glass. Thus if the object is mounted in Canada balsam under a cover glass, and the gap between the cover glass and the front lens of the objective is filled with cedar-wood oil there is no undue aberration at the plane surfaces even for the most oblique rays. The second surface of the front lens can work under "near-aplanatic" conditions, and the convergence of the rays can be increased by a thin meniscus lens of the type illustrated in Fig. 7. The residual chromatic under correction has to be compensated by the chromatic over correction of the following doublet or triplet lenses, but the amounts of spherical aberration can be kept relatively low.

It was explained above that, by the use of doublet lenses with glasses of different dispersive powers, the red and blue rays of the spectrum can be brought to a common focus; the relative distribution of the focal positions for other wave lengths is of the type shown in Fig. 30 (achromatic lens curve); the focus for the yellow-green part of the spectrum lies nearest to the lens. The use of the substance "fluorite" as a kind of crown glass of very low dispersion even in one of the parts of a microscope objective enables important improvements to be made in the spherical and chro-

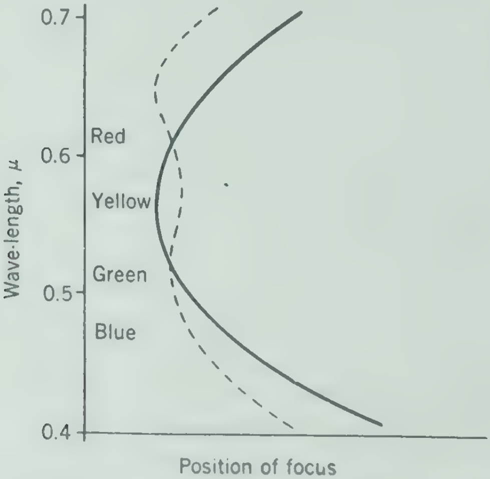

FIG. 30. Residual chromatic aberration. Solid line: achromatic lens. Broken line, apochromatic lens.

matic correction, though it is less satisfactory than glass in the mechanical sense and more liable to damage. By the use of still more complex design, including fluorite with other glasses of special types; it is possible so to correct the chromatic aberration that there is (as it were) a double fold in the curve of focal distance against wave length, Fig. 30, and moreover the spherical aberration may be corrected for two colors. Such highly corrected lenses were called "apochromatic" by their originator, Abbe. They include the best "dry" as well as immersion lenses, and they have rendered possible important advances in the microscopy of difficult objects.

The chromatic and spherical correction of microscope objectives for the axial object and image points is not the only thing requiring attention. "Coma" must be removed from the off-axis image points; and the relative flatness of the field is a matter requiring attention in design, otherwise the field may be in focus only over a small annular zone at one time. While perfection in the latter respect is hardly attainable in ordinary high-aperture lenses, some approach to "astigmatic over-correction" is usually sought. Another matter is the preservation of a constant magnification for the different colors of the spectrum. Apochromatic objectives are liable to give some trouble in this sense, but fortunately the effects can be removed by a suitable design of the "compensating" eyepieces used with them.

In some types of objective a more or less usual construction can be supplemented by auxiliary lenses (in which some of the principles of photographic lenses are used) to give specially wide and flat fields in focus at one time; this is of advantage in photomicrography.

The eyepiece has a function similar to that of the magnifier; but the conditions differ, because the light from any point of the image to be magnified is contained in a very narrow bundle of rays. Satisfactory results with achromatic objectives are obtained by the Huygenian eyepiece, Fig. 31 (A). A white ray is dispersed by the first (or "field") lens so that, for example, the blue ray meets the eye-lens nearer to the axis than the red. The design is such that the parallel bundles of colored rays enter the eye at the same angle so that the retinal images have the same size. Images are thus without the colored fringes which would result from the use of a simple lens as a magnifier; moreover the design of the eyepiece facilitates some correction of the curvature of field in a manner not attainable with a single lens. The angular size of the field attainable with Huygenian eyepieces is small, about 40 to 50°, but a great variety of types using more complex designs is available; they offer various advantages i.e., perhaps improved corrections, or a wider field up to 70° or more, or a larger eye clearance. The use of compensating eyepieces with apochromatic lenses was mentioned above.

The selection of the objective usually turns on the required degree of

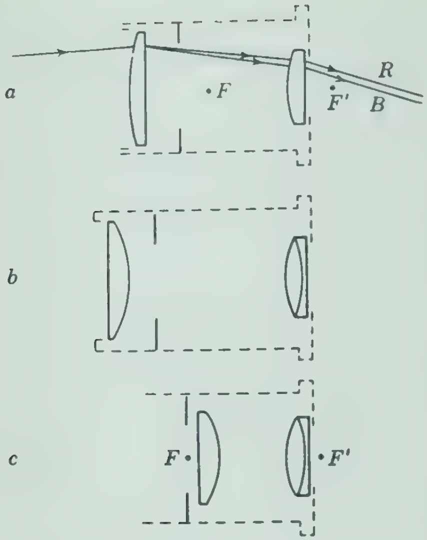

Fig. 31. (a) Huygens eyepiece. (b) Compensating eyepiece. (c) Achromatized Ramsden eyepiece.

resolution, the appropriate *NA* being chosen with an achromatic or apochromatic system according to the purpose of use. For example if a *search* is being made for the presence of an easily recognizable object with an image of high contrast, an achromatic lens will serve; on the other hand if a known object is being examined for details of fine structure an apochromatic lens may be essential.

The selection of the eyepiece will usually be made, first as regards the focal length in order to secure such a magnifying power as is required to secure easy recognition of the objects or details expected. The type of eyepiece will depend on such factors as the required width of field; for example if the whole of a specimen in a slide has to be searched for a micro organism, the magnifying power will be cut down to the minimum necessary for definite recognition and the width of the field will be increased as much as possible consistent with retention of adequate definition. Some workers may require spectacles with the instrument; they will find an eyepiece with a wide eye-clearance helpful.

3. Working Distance

It should be clear from the discussion above that the permissible working distance of an objective is usually fixed by optical considerations, within comparatively narrow limits. Each lens is designed for a standard thickness of cover glass (commonly 0.18 mm) and any variation of the image-space conjugate will introduce appreciable spherical aberration. The working distances (the gap between the first lens and the cover glass) given in the table below are those cited for objectives made by Messrs Cooke, Troughton and Simms Ltd., York; they are fairly typical.

Type	Focal length, mm	NA	Working distance, mm
Achromatic, dry	0.25	0.15	17
Achromatic, dry	16	0.28	5.0
Achromatic, dry	4	0.65	0.71
Ahcromatic, dry	4	0.85	0.37
Achromatic (Immersion)	1.8	1.30	0.12
Apochromatic (Immersion)	2.2	1.32	0.12

The use of a cover glass with excessive thickness produces appreciable spherical over correction, and vice versa, which it may be important to correct. One way of doing this is a slight alteration of the back conjugate distance, a few millimeters at a time one way or the other until a sensitive test (see below) shows that the spherical aberration is corrected. Some objectives have been made with "correction collars," allowing a slight adjustment of the distance between two component lenses to produce a similar effect without alteration of the "tube-length."

The subject of working distance cannot be dismissed without mention of the modern possibilities of enlarging the distance by the use of reflecting objectives, in which the control of wide-angle cones of rays is possible without introducing the chromatic aberration inseparable from refraction. A reflecting attachment due to Dyson (1949) is illustrated in Fig. 32. The object is situated in an equatorial plane of a spherical reflecting surface which would project the image of the center M into the same point. However, a semi-reflecting surface AB is interposed, and a real image of M is formed at M′ near the pole of the spherical surface. A small opaque screen E prevents direct rays from reaching the objective. In practice the reflecting parts are carried in a holder attached to the objective, and the space between the object and the reflector AB can be comparatively large; for example in a model made by Messrs R. and J. Beck Ltd. (London) a working distance of 12.8 mm is obtained while using NA of 0.574. Such reflectors have some obvious drawbacks, but in the examination of hot objects etc, where a long working distance is essential, they have great advantages.

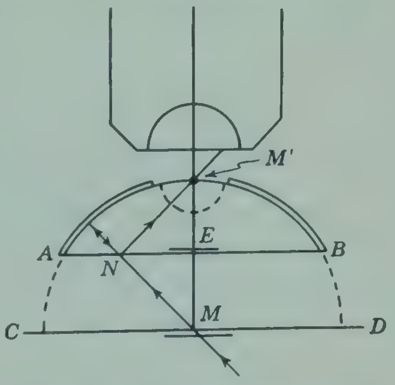

FIG. 32. Dyson's device for increased working distance (diagrammatic).

4. SUB-STAGE AND OTHER CONDENSERS. DARK-GROUND ILLUMINATORS

The function of the condenser was discussed above; the optical design of the systems resembles a more or less crude objective reversed. Figure 25 shows a type uncorrected for chromatic error, and Fig. 26 an achromatic type. The observation of different objects requires a careful control of the illuminating light, and there are circumstances in which it may be helpful even to use an actual objective (reversed) with the object mounted between two cover glasses (but only when the very greatest freedom from stray light is essential for the observation of some image with faint contrast). The "Abbe" condenser (an inexpensive two-lens type) has much spherical aberration, and uniform illumination of the field cannot easily be obtained except with a fairly large source, of which the image is formed somewhat above the plane of the object. The control of the light is difficult and a better performance can be obtained with an "achromatic" condenser. If the *illumination* is to have NA exceeding unity, the condenser must be oiled on to the slide; but very often the unit of NA is as far as the illumination can be taken without loss of contrast.

A "dark-ground" illuminator, Fig. 33, passes an annular cone of light through the object, the minimum NA of the cone exceeding that of the objective by a small margin. The object is then "seen" entirely by diffracted and scattered light. Dark-ground illuminators, Fig. 34, are also made for opaque objects, the light reaching the object from around the objective. The makers' catalogues should be consulted for details.

5. THE MECHANICAL CONSTRUCTION OF THE STAND

In early microscopes, the optical parts were mounted in a "body tube" which itself slid inside the outer "body," the latter being supported perhaps by three "limbs" rising from the base. In a simple modern instrument, Fig. 35, the "body" (B) has shrunk to the member which separates the

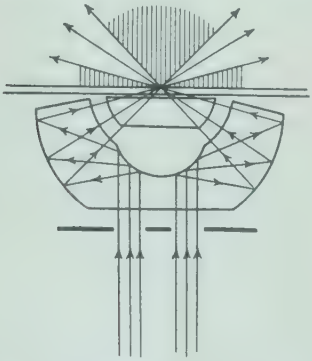

FIG. 33. Focussing dark-ground illuminator (Beck type).

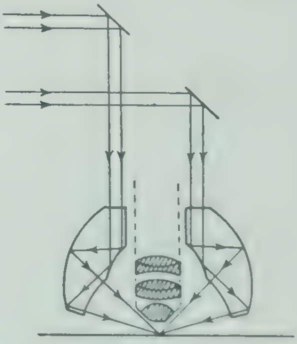

FIG. 34. Ring illuminator for opaque objects.

one massive limb (L) from the body tube; it usually articulates with the body tube in a slide with the rack-pinion motion of the coarse adjustment (C), and articulates with the limb in a (parallel) slide controllable by the fine adjustment movement (A).

The stage (S) is usually rigidly fixed to the lower part of the limb. If the objects for study permit of a sloping stage, the limb may be pivoted into the base or foot (F) of the instrument so that the optical axis can be inclined at a suitable angle for comfortable observation. However, if a prismatic eyepiece, itself permitting a suitably inclined direction of view, is to be employed the pivot can be omitted.

The substage condenser, fitted with a suitable focussing slide, is mounted below the stage and otherwise moves with the limb; a centering adjustment is seldom necessary for ordinary work, but it is very desirable that the condenser shall be easily withdrawn from the optical train. A suitable swinging movement can usually be operated when the condenser is lowered from its operating position. It should not be possible for the top lens of the condenser to make actual contact with a slide in position on the stage. An iris diaphragm controls the aperture of the condenser, and it is helpful to have auxiliary holders for light filters and other screens which may be required. The tail piece carrying the mirror (M), is the lowest attachment to the limb.

The base like the limb, is of massive construction to avoid vibration, and normally rests on three feet. Its dimensions and design should be such that

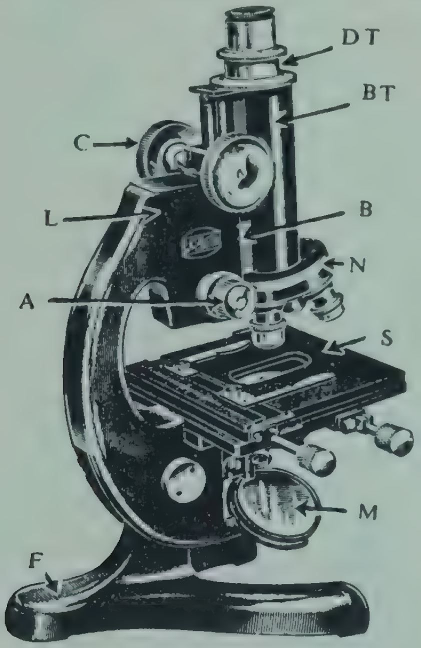

FIG. 35. Monocular microscope (R. & J. Beck, London).

the optical axis can be made horizontal if necessary without instability of the whole instrument.

The rack and pinion movements of body tube and elsewhere need no detailed discussion. Care should be taken to see that their "sweetness" of movement arises through good workmanship and not from thick grease. Many forms of fine adjustment are in use; a lever movement is very often employed; no appreciable "loss of time" or back-lash should be present. The operating heads should be divided so that the movement can be readily read off in microns. Intervals of 0.2 microns or thereabouts should be recognizable.

The body tube is fitted at its lower end with the objective-holding screw fitting (R. M. S. standard; internal diameter 23.3 mm, 36 threads to the inch) and there may be a revolving nose piece (N) or other mechanical device, for changing objectives rapidly; the upper end carries the adjustable draw tube (DT), usually fitting well enough to have a stable position under the weight of whatever eyepiece or eyepiece system may be in use, but adjustable without undue force, and graduated in millimeters. If a worker gives great attention to draw-tube adjustments he may find it helpful to select a model in which a rack and pinion or coarse-screw movement is provided for this purpose.

The mechanical tube length is the projected axial distance between the shoulder of the objective mount and the upper rim of the draw tube against which the shoulder of the eyepiece comes into position. A normal magnitude for this interval is about 160 mm, and in modern times the objective and eyepiece systems of one maker are often so designed and fitted that they are *parfocal*, i.e., they can be freely interchanged without a total loss of focus. The "optical tube length," being defined as the distance between the second principal focus of the objective and the image point, varies to some extent from one objective to another, but the maker's catalogue will cite the magnification valid for the normal working distance.

Prismatic eyepieces (sometimes used merely to obtain an inclined direction of view—but essential in binocular systems) usually need an extended optical tube length, which is obtained by the use of a suitably corrected negative lens mounted below the train of prisms (Fig. 36). The magnification will be increased by this. A binocular eyepiece must incorporate an efficient adjustment for the inter-ocular distance to suit the observer. There should be independent focussing for one eyepiece, just as in a prismatic binocular or field glass, to allow for anisometropia.

The stage may be of square or circular form. In the simplest case the slide is held by spring clips and moved by hand in order to bring various parts of the object to the optical axis, but this suffices only for very low-power work. A mechanical stage by which independent movements in two

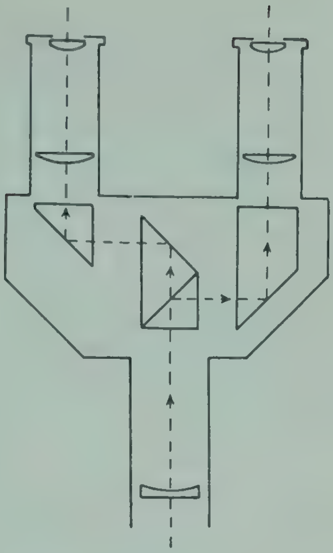

FIG. 36. Principle of prismatic eyepiece system; the arrangements for inclining the axis and adjusting the inter-ocular distance are not shown.

perpendicular directions (measured by verniers) can be given to the slide is usually essential, and may be either "built-in" or independently attached. A rotating circular stage with centering adjustments will allow certain objects to be put "the right way up" when this may be important, and will be essential if observations are ever to be made with polarized light.

The foregoing remarks are relevant only to the stands of the types commonly used by biologists. 'Research microscopes' of a great variety of patterns offer many variants, e.g. focussing by movement of the stage etc. If the design of the average instrument appears to be stereotyped it must be remembered that it has evolved as the result of much experience and many essays in improvement. There is no space in the present account fully to describe the many modern varieties of illumination, especially those for opaque objects. Phase-contrast observations are dealt with elsewhere in this book.

6. Accessories for Photomicrography

The standard optical equipment for photomicrography differs in two main respects from that for visual observation. Firstly, a special "projection eyepiece" is preferably used in which the distance between the components may be adjustable according to the final distance between this lens and the image; it is also fitted with a diaphragm in the plane of the exit-pupil to control stray light. Secondly a source of light of high luminance is used in order to reduce the exposure.

The mechanical accessories afford some means of rigid support for the camera (bellows and plate holder) and can be chosen to work with the microscope axis either vertical or horizontal.

Specially designed camera units with an adaptor fitting into or over the upper end of the standard eyepiece tube have become popular. The eyepiece carries an auxiliary lens and prismatic reflector which enables the image to be observed visually until the photograph is about to be taken, when the reflector can be displaced and the exposure made with the aid of a cable control. The magnification obtainable with such a camera is roughly the quotient of the camera length divided by the focal length of the eyepiece used for projection. A precise measurement of the magnification is, however, best made by photographing a "stage micrometer," i.e. a set of ruled lines (perhaps one millimeter divided into tenths and hundredths) suitably mounted in a microscope slide.

V. Practical Manipulations

1. Choice of Objective and Eyepiece

In cases where the size of high-contrast object detail to be resolved is known the *minimum* necessary Numerical Aperture can easily be calculated from Eq. 14. However, the following table, based upon practical observations, will be found to represent the average performance of well corrected objectives.

NA (Visible light)	Resolving limit (microns)	Approximate magnification advisable	Lines per inch corresponding to resolving limit
0.1	3.39	100	7,500
0.3	1.13	300	22,500
0.6	0.56	600	45,000
0.9	0.38	900	67,500
1.2	0.28	1000	90,000
1.4	0.24	1400	105,000

In cases of very weak contrast, extra magnification may be required. The makers usually specify the magnification of the objective when used at the prescribed tube length, and the eyepiece magnification is selected to bring up the total magnification (the product of the two values) to the required amount.

In other cases the resolution in the image may not be of much importance in comparison with the necessity of obtaining a complete image of a certain object, or some other requirement. The limits of performance of the eye, the curvature of the image field etc. and many other considerations enter into play. Practical trials coupled with some knowledge of the limits of possibility, will usually suggest an acceptable arrangement.

2. SETTING UP THE INSTRUMENT FOR OBSERVATION

The over-riding aims of the procedure described below are as follows:

(1) To obtain adequate illumination of the required area of the object so that each point of it effectively radiates uniformly into the required range of numerical aperture.

(2) To restrict the light passing through the object to the minimum necessary, thus avoiding glare, etc.

(3) To obtain effective control of the conditions of illumination.

It will be supposed that the source to be used has a diffusing surface. The most usual type for visual work employs an opal bulb lamp in a suitable housing; the aperture is controlled by the "lamp iris." To prepare for observations with a high-power dry objective the stand is placed on a firm bench of such a height that the observer's head can have a comfortable inclination when the eye is close to the eyepiece, and an adequate tilt is given to the axis if required. The lamp is placed opposite the mirror (plane side uppermost) of the microscope so that the lamp iris is about 6 inches from the mirror surface, and the following procedure is carried out.

a. All the 'optics' are removed from the path of the light (with the exception of the eyepiece prism system and associated auxiliary lens if employed), the sub-stage condenser being lowered and swung aside. The mirror is tilted until the beam from the lamp passes freely up the tube.

b. Place the object on the stage and insert a low-power objective (1 in. or 2⁄3 in.) and a ×10 eyepiece. Focus with the coarse adjustment.

c. Swing in the substage condenser. Close down the lamp iris and rack up the condenser until the image of the lamp iris appears in focus with the object. A pencil point moved about near the lamp iris may help to recognize the required adjustment.

d. Remove the eyepiece and close down the substage iris. Place the eye central at the end of the tube and observe the image patch representing the substage iris. Center it by the centering screws if provided (otherwise ascertain that its centering is adequate). The patch should appear concentric with the back of the objective, and should be evenly illuminated if the mirror adjustment is correct.

If it is necessary to replace the low-power objective by a high-power dry lens, insert this objective, re-focus, remove the eyepiece and repeat "d."

e. Before inserting the eyepiece, gradually open out the substage iris until its image patch has a diameter about 2⁄3rds that of the objective aperture.

f. Replace the eyepiece and re-focus the image. Adjust the size of the lamp iris until the illumination is confined to the area of the object to be observed. The illuminated area may be centered in the field by a small adjustment of the mirror. Remove the eyepiece and check that the "patch" is still properly illuminated and central. Replace the eye-piece and make any small adjustment to the substage necessary to obtain the best compromise between contrast and resolution.

Note that if an Abbe condenser is used, its aperture should be closed down for c above, but it will have to be racked upwards to some extent to obtain the even illumination of the 'patch' in the operations d, e, and f.

Extra care is needed with an immersion lens. A substage condenser will pass an illuminating cone of adequate aperture (for most objects) through a layer of air, but there will be much reflection and tendency to scattering which can be reduced by putting a drop of cedarwood oil on the top lens of the condenser before racking it up to focus the image of the lamp diaphragm. If the condenser is properly designed and the thickness of the slide is normal, the oil drop will be squeezed out into a thin film. The preliminary steps of the setting-up, a–d, are otherwise as above.

Let us suppose, however, that the immersion lens does not belong to the parfocal series, so that we cannot simply swing it into position after observations with the dry lens. A drop of immersion oil (thick but not "tacky") is placed on the front lens and the body tube racked up well until the immersion lens can be placed into position on axis. The observer, watching from beside the instrument (perhaps with a hand magnifier) now racks the body-tube down until the front lens of the objective is nearly but not quite in contact with the cover-glass, the oil film having spread out between them into a layer less than about a tenth of a millimeter thick. The aperture of the condenser being kept fairly small, he now watches for the image using the low-power eyepiece while the lens is cautiously moved *upward* by the fine adjustment until the image is seen; the condenser aperture is opened up as before and the high-power eyepiece brought into play. Attempts at focussing by moving the objective downward are only apt to result in a broken slide. A very practiced hand can sometimes use the coarse adjustment to find the image; but it is easy to overshoot the mark. Having found the image, repeat adjustments d to f.

The principles illustrated in (2) above will give the clue to the procedure desirable in many other cases which cannot, for lack of space, be described here. The importance of correct control of the *illumination* cannot be over emphasized, and the inspection of the back aperture of the objective should never be omitted. When a dark-ground illuminator is employed, the procedure is very similar, but a source of high luminance (point o'lite or arc lamp) will usually be required. The aperture of such an illumination will be fixed by its construction, and the inspection of the back of the objective when focused should not show any *direct* light. The makers of the illuminators usually provide detailed instructions for their employment.

3. Use of Various Sources of Light

When setting up apparatus for photomicrography with a small source of high luminance, such as a coiled filament lamp, the remarks under III,5. should be borne in mind. The manufacturers of microscopes often provide illuminating units containing such a lamp with a suitable auxiliary condenser, and holders for diffusing discs or color filters.

In setting up, the lamp and auxiliary condenser are now arranged so that a sharp image of the filament is thrown into the plane of the substage condenser, the plane mirror being adjusted as before (without the microscope optics) so that the light passes symmetrically up the tube. A diffusing disc (very finely ground glass or etc.) should now be placed in position so that the substage aperture is uniformly illuminated.

The use of the diffusing filter should only be omitted if the source (perhaps the crater of an arc lamp) is so uniform that the sub-stage aperture is completely and evenly illuminated; and the auxiliary condenser is not giving a poor image of the source through too much spherical and chromatic aberration. In the latter case the diffusing filter will help to avoid the annular shadows which might be given by an unsuitable lens. An auxiliary condenser should be well enough corrected to avoid troubles of this kind.

The use of the diffusing filter will scatter a good deal of light outside the substage aperture; this cannot get into the microscope to weaken the image contrast, but the penalty is paid in diminished intensity of the image. If a very short exposure is essential a ribbon filament lamp or a carbon arc may be employed and the diffusing filter omitted. A fairly high-intensity extended monochromatic source is given by a high-pressure mercury arc in a suitable housing and used with a special green filter.

4. Photomicrography

If the field is too bright for comfortable vision while using a source of high luminance, a neutral or other filter of suitable density should be placed (preferably near the auxiliary condenser aperture) in the path of the light for *all* the preliminary visual adjustments, which must be carried out with the same source. It is easy to misjudge the relative aperture or uniformity of the illumination at the back of the objective if the intensity is too great. The filter is only removed for the actual photography.

The projection eyepiece being placed in position, the image is focused in the intended plane of the photographic plate or film, but preferably *not* with a ground glass screen. It is better to use a screen of good quality glass and view the image in the plane of the plate by a fixed-focus magnifier mounted in a short tube so that its principal focal plane is represented by the end of the tube.

The exposure is best judged by exposing a trial plate in strips of increasing exposure. When working with different magnifications etc., it may be helpful to remember that the illumination of the image plane is directly proportional to the square of the numerical aperture of the illuminating beam and inversely proportional to the square of the magnification. Hence the exposure will diminish approximately with the square of the effective condenser *NA* and increase with the square of the magnification. These remarks apply only to the ordinary cases and not immediately to the dark-ground or reflecting systems.

Many important points of photographic technique are beyond the scope of these notes.

5. TESTS ON OBJECTIVES AND EYEPIECES

The objective of a microscope is the most vital part of the whole system. The lenses produced by makers of repute can usually be relied upon, but in times past the output of some of the smaller factories was of very uncertain quality. The modern maker may issue a certificate of interferometric testing, or such tests can be made by well-equipped standardizing laboratories. Failing these, tests are best made with some standard object such as the Abbe test plate—or a "star" test in which a "pinhole" in a silver film on a slide is brightly illuminated by the sub-stage condenser and made to give a beam filling the whole aperture of the objective. The residual spherical aberration of the lens is judged by the perfection (Airy disc) of the pinhole image, and also the absence of marked differences between the expanded patches inside and outside the focus (see Martin, 1950). The chromatic correction also is estimated by the appearance of the extra-focal images.

Observations with so-called test objects such as diatoms etc, should only be trusted by workers of much experience, since objectives afflicted by much zonal aberration can, on occasion, produce images showing marked contrast and a generally attractive appearance. The whole matter of testing requires a long apprenticeship.

All lenses should be inspected periodically with the aid of a magnifier for cleanliness, freedom from scratches, firmness of the lenses in the mounts, and freedom from "starring" in the cemented components.

Owing to the narrow aperture of the beams passing through an eyepiece, the optical requirements are not nearly so severe as for objectives, and after the cleanliness, mechanical stability, and perfection of polish, the principal matters for inspection are the oblique chromatic correction, the adequate distance of the eye point, and the freedom from the formation of shadows in the field of view under ordinary conditions of observation.

Ordinary Huygenian eyepieces have an interior field diaphragm, and show a blue rim to the edge of the field when the eyepiece is removed from the instrument and held up to the sky. They can be used effectively with low- and medium-power achromatic objectives. Compensating oculars show a red or yellow-orange rim under a similar test. They are intended for use with fluorite and apochromatic lenses and will be preferable with some high-power achromatic lenses. The use of a properly compensated eyepiece produces freedom from colored borders to high-contrast images away from the center of the field.

VI. The Interpretation of the Image

1. Study of the Images Corresponding to Various Objects, Including Variations of Opacity and Optical Path in the Object Region

The majority of biological objects are of a semi-transparent character, conveniently viewed by transmitted light when they are evenly illuminated. Under certain circumstances it would be possible to predict more or less exactly what kind of image would be produced by a given optical system from a given object. Consider for example a "high-contrast" object consisting of an opaque lamina of infinitesimal thickness containing holes of certain shapes. The name of "amplitude object" can be conveniently used. A perfect optical system would produce an image in which certain evenly illuminated areas possess shapes exactly similar (geometrically) to the holes; but an actual system is imperfect, for it has a limited numerical aperture, and even if the "holes" are self-luminous each infinitesimal point will be imaged by an "Airy disc" concentration of finite size. The result is that the images of sharp edges are gradated, and corners are more or less rounded, the "geometrical-shape" error being relatively the more severe the smaller the object. It is not essentially a difficult matter to predict the image from knowledge of the object, but the exact solution of the converse problem is in general much more difficult, especially when the object-size approaches the resolving limit of the system. For example if a very small corner of a crystal in the object plane has an image with a rounded corner, it is immensely difficult to determine the degree of residual rounding actually present in the crystal itself.

However, given an amplitude object of the above type, *and an objective really free from aberration,* it is possible to say that the aperture of the illumination should be approximately equal to that of the objective for optimum performance; the object then behaves as if it were self-luminous for most practical purposes, and full use is made of the resolving-power of the system; the maximum geometrical similarity between the object and image, possible for the system, is obtained. Few objectives are, however, so perfect as to admit the use of such wide-aperture illumination, and stray light may cause trouble.

A low-contrast object such as a lamina containing a geometrical pattern of areas of different transmissions can be regarded as a high-contrast object *plus* more or less evenly illuminated background. It is still an "amplitude object." The contrast in the image reproduces, ideally, that of the object, and the same optical requirements apply. A colored object-lamina is one with selective contrast according to the wave length of light, and again the color pattern should, ideally, be reproduced in the object. In all such cases an *ideal* objective would allow the use of an illuminating cone of full aperture. If, however, there are residual aberrations, a reduction of the aperture of illumination has an effect in sharpening the image, similar in one way to the use of a small stop in a camera lens, though there are other effects concerning the phase relations of direct and diffracted light.

Few objects, however, consist of an infinitesimally thin lamina of varying transmission. An unstained section made by a microtome or, say, a film of sputum containing living organisms has to be regarded as a lamina of finite thickness containing local three-dimensional variations of refractive index. It is often without appreciable absorption of light which could render the details visible in the way discussed above.

How is it that local differences of refractive index in a medium can be "seen"—as for examples when syrup is being stirred into water? The filament of higher refractive index refracts some light, perhaps outside the pupil of the observer's eye, and is thus seen dark on a lighter background. But in the microscope the use of a high aperture objective would tend to defeat this effect, because light suffering *small* deviations will still be brought to the image and the detail will disappear. Hence there is a conflict between the hopes of maintaining high resolution and, at the same time, adequate contrast in such an image. One possibility of rendering such structures visible is by the use of dark-ground illumination, where the background light is eliminated, and the details are seen by refracted, diffracted, or scattered light though indeed the appearances are often hard to interpret. Roughly speaking, the regions of illumination in the image correspond to regions of a high gradient of refractive-index change in a direction perpendicular to the light—or a sudden variation of transmission. Thus a globule of fat in water would probably be represented by a narrow ring of light where the refractive index of the medium changes from 1.33 to about 1.42. But the use of dark-ground is particularly liable to give unexpected 'fringe' and other optical effects which may not correspond to actual physical structures in the object.

When ordinary illumination is employed the occurrence of changes of refractive index across a border "perpendicular to the slide" will give characteristic appearances. Referring to Fig. 37, total reflection of the less oblique illuminating rays occurs if there is a drop of refractive index, but

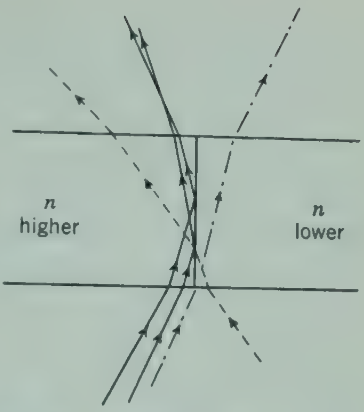

FIG. 37. Origin of Becke line.

the rays incident in the reverse sense are transmitted. In consequence of this all such rays encountering the border tend to emerge on the side of higher index. In many cases a bright line is seen (the Becke line) which, though not apparent when the microscope is focused on the object, develops and moves away from the boundary into the high index medium as the microscope objective is raised. The reverse effect is noticed as the tube is lowered.

Other characteristic appearances are seen when lenticular or more-or-less spherical bodies are suspended in media of refractive indices differing from their own. In the case of a sphere of relatively high refractive index the rays of an incident parallel bundle are subject to marked spherical aberration after refraction; see Fig. 38. When the microscope is focussed on the plane F_0 containing the equatorial diameter the exterior outline is sharply defined, but the loss of refracted light (some of which may miss the objective) gives a dull illumination to the inner part of the image. However, if the focus of the microscope is shifted to F_1, most of the refracted light entering the objective is now apparently contained within a ring (of radius corresponding perhaps to BR_1 in the figure) of diameter much smaller than that of the sphere. A further shift of focus outward to F_2 shows most of the light within an even smaller disc (radius CR_2). The dark out-of-focus image of the sphere has thus a bright center which shrinks as the focus is moved "upward." A reversed set of changes occur if the sphere (perhaps a bubble) is of lower index than the surrounding medium.

Broadly speaking, the "stained" object should respond well to relatively high apertures of the illuminating cone, but even in such cases there are

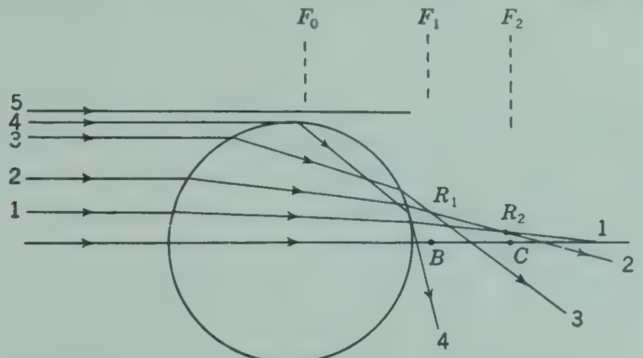

FIG. 38. Optical action of refracting sphere.

parts of the structure which do not stain, and still have to be seen by reason of their difference of refractive index from that of the embedding medium. It is, moreover, true that the complex processes of staining may profoundly alter the relative forms of certain structures so that some means of dispensing with staining would often be welcome. It is the general experience that refractive-index-change (or "phase") objects are commonly seen better in the microscope when the aperture of the condenser is drastically reduced, and it is essential to understand the reason for this. Imagine a 'grating' such as might be found in a diatom—exceedingly fine parallel rods of silica now embedded in a mounting medium of higher refractive index; it typifies a periodic "phase" object. If the *NA* of the illumination is small the light is largely "coherent," and various diffraction beams of different orders spread out at their various angles with the axis.

It has been shown, theoretically, that as the objective takes up more and more diffracted beams by increase of its numerical aperture the pattern of phase (or relative timing) of the vibrations of light in the object plane is represented more and more nearly by the phase pattern in the image; for example the vibrations in the images of the silica rods would be ahead of those in the images of the spaces. This is the only difference in the image region, and it cannot be 'seen' by the eye, which cannot detect mere differences of timing. However, an important change can be made by putting the image slightly out of focus, because this upsets the relative phases in the diffracted beams as compared with the central beam. The complete argument cannot be followed here, but an image is obtained which has indeed a main pattern of intensity variation corresponding to the spacing of the object, but from which the relative width of "rods to spaces" cannot be truly deduced.

A similar action occurs when an objective has more or less spherical aberration; the variations of phase thus given to the diffracted beams may produce quite a 'contrasty' and attractive-looking image, but giving a picture false in important respects. Phase-contrast (dealt with elsewhere in this handbook) is essentially a method in which a single-valued phase-change is given to *all* the diffracted light; under certain conditions the image will then be much more truthful; for example in our case of the grating the relative intervals of the rods and spaces will be more truthfully indicated.

The reduction of the aperture of illumination to obtain increased visibility of "phase objects" entails some degree of loss of resolution for amplitude objects, but even if the illumination aperture is reduced to zero (parallel beam illumination), the resolving limit in representative cases is only increased by a factor less than 1.5 (Hopkins and Barham, 1950). Also, Hopkins has shown that if the object is a small opaque disc, coherently illuminated, the lower limit *d* of diameter required for reasonably sure *recognition* in the image (as a mere darker spot, not significant as to relative size) would be given by

$$d = \frac{0.21\lambda}{NA},$$

where NA is the numerical aperture of the objective. This is of interest to compare with Eq. (13) above.

The corresponding formula for incoherent illumination has not yet been worked out, but there is no reason to think that the value of d would be *very* much smaller.

To sum up, the ordinary microscope should be perfectly satisfactory for all the numerous varieties of "amplitude objects." The more highly corrected the objective, and the higher the possible NA of illumination, the higher the degree of geometrical truth in the image. Phase objects and mixed phase-and-amplitude objects cannot in general be imaged with high contrast and visibility, and (at the same time) the highest degree of resolution and geometrical faithfulness in the ordinary microscope. This is the proper field for "phase contrast" and interference microscopes, but there are still many purposes for which even an imperfect image of a transparent structure may suffice for its recognition and rough measurement; if its exact structure is to be investigated, these other methods are now obligatory.

2. Effects of Aberrations

The short discussion in this section is confined to the effects of the chief aberrations of the objective in their relation to the interpretation of the image.

Residual axial chromatic aberration of the objective encountered in "achromatic" lenses gives an effect of slight coloration even of the images of completely colorless objects. The image of an edge of an object may appear tinged with a light apple green just outside the best focus, and with the complimentary color (light purple) just inside. Differing "shades" of three colors indicate differences in the chromatic correction of various lenses. *Apochromatic* lenses should show no recognizable color effects apart from those due to the object.

As already mentioned, colored fringes to images in the outer parts of the field indicate a chromatic difference of magnification which may in principle be removed by the correct choice of the eyepiece. Unsymmetrical color in the center of the field may indicate a lack of centering in one of the components of the objective. It should be noted that color effects in the images of periodic structures may be due to diffraction, and have no relation to the aberrations of the system.

Spherical aberration of an objective will reduce the contrast in the detail of amplitude objects, and will set a limit to the numerical aperture of the illuminating cone consistent with good contrast. It prejudices the "truth" of the image detail in a geometrical sense. It may be recognized by the characteristic lack of symmetry between the extra-focal images of pinholes or opaque edges.

Apart from the residual errors in design or construction of an objective, it may exhibit spherical aberration if used at the wrong tube length—or with an incorrect thickness of cover glass. As already mentioned the tube length can sometimes be changed to produce more-or-less compensation for the cover glass. Apochromatic lenses are corrected for spherical aberration in two colors, and require very carefully chosen conditions to enable them to give their optimum performance.

The monochromatic extra-axial aberrations, *coma*, *astigmatism*, and *curvature of the field*, produce characteristic effects in the definition of the outer parts of the image, but do not necessarily lead to difficulty in the interpretation, since, at least in visual observation, the object can be moved into an axial position. If the lack of symmetry associated with *coma* appears in the center of the field, it may be due to a tilting of one of the objective components, and this is a matter for the attention of the maker.

3. Spurious Resolution and Artifacts

Objects with inner periodic structures, like diatoms, if illuminated with a fairly narrow cone, owe the 'resolution' of the image to interference effects between various diffracted beams—and such interference may occur in widely differing settings of the focus. Especially with such 'phase objects' as these, the phase relations for maximum contrast can occur at a focal setting quite perceptibly different for that producing the best image of the outline of the object (essentially an amplitude object). The periodic image is then not a true image of the original structure, since local details may be suppressed. However, it can usually be interpreted as *due* to a real periodic structure in the object, and used to derive the average spacing even in the absence of a one-to-one correspondence.

Spurious resolution can even occur with high-contrast amplitude objects, though these are comparatively rare amongst microscopic objects. Abnormal methods of illumination (dark ground, oblique beams, etc) can, however, occasionally produce sets of realistic looking "fringes" which can be mistaken for genuine images of some object structure, especially with some kinds of phase object. Spurious color effects can be due to the difference of angles of diffraction for the various components of white light, some being lost in the image.

Careful investigation is necessary before the reality of such apparent "objects" can be confirmed. Pure diffraction 'fringes' are enhanced by the use of special monochromatic light (such as mercury green) in place of white; the spacing of fringes is, moreover, smaller with blue than with red light.

If a genuine structure is suspected, efforts must be made to observe it with varying conditions of illumination. Naturally the degree of certainty differs with the actual observation. For example, the *presence* of some fine filament extending from a microorganism may be indubitably inferred from a dark-ground image; the measurement of its true diameter presents a problem of entirely different order.

4. Empirical Studies

It will be evident from the foregoing remarks and discussions that the interpretation of the microscope image presents much difficulty, especially in the case of "phase-objects." Even when the theoretical problem of the refraction, diffraction, and scattering of light by various objects can be worked out, the prediction of the image is complicated. The reverse procedure is much more uncertain. Much can be done in avoiding the more obvious pitfalls by empirical studies of artificial objects of known physical structures. Comparatively 'coarse' objects of this kind, when imaged by lenses of low numerical aperture under various conditions of illumination, may present various purely "optical" effects similar in many ways to those encountered in the images of much finer objects. Empirical studies of this kind employing optical models may be of help in problems of interpretation. Unfortunately, however, it is not readily possible proportionately to enlarge the wave length of light for the experiment—at least with ordinary apparatus. The method cannot therefore throw much light on diffraction and interference effects.

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Chapter 7

PHASE AND INTERFERENCE MICROSCOPY

H. Osterberg

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I. Purposes of Phase and Interference Microscopy

1. IMPROVEMENT OF CONTRAST

Contrast in the sharply focused images of unstained particles can be increased by either phase or interference microscopy. The possible gain in contrast becomes marked when the optical path[1] difference between particle and surround falls below one-twentieth wave length.

The phase microscope is most effective in augmenting contrast with respect to areas over which the optical path varies rapidly. Such variations occur in the vicinity of small particles or near the edge of large particles. Consequently, the entire area of small particles and the area near the edge of large particles can be seen with improved contrast in the phase microscope. The central area of large plate-like particles assumes the same brightness as the image of the surround whenever the particle has negligible absorption.

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[1] Optical path is the product of refractive index and thickness.

The interference microscope is most effective in demarcating areas having like optical paths. These areas appear as fringes or zones of like brightness or darkness. Large plate-like particles appear uniformly bright or dark in the interference microscope.

The principles of phase and interference microscopy can be combined to obtain greater contrast than is possible by either system alone. This combined method becomes, in principle, so sensitive to small differences in optical path that its utility is restricted by the ultimate irregularities of the polished surfaces between which the object specimen is confined.

2. Measurement of Optical Path

The multiple beam interference microscope, operated under the conditions of narrow-coned axial illumination, is well suited to the measurement of optical path difference between particle and surround. These measurements are particularly simple with large particles whose variation in optical path is gradual and amounts to several half wave lengths. The image of such particles displays a family of interference fringes. Successive bright or dark fringes correspond to an increase or decrease of one-half wave length[2] in optical path. When high precision is not required, it suffices merely to count the number of bright fringes as number of half wave lengths of optical path. The illumination is monochromatic. The interferometer plates are preferably adjusted for parallelism.

If the plates of the interferometer are adjusted off parallelism to form straight interference fringes within the field of view, the lateral displacement of these fringes at the particle affords a direct measure of optical path differences that are a small fraction of one wave length. This method is identical to that discussed, for example, by Tolansky for measuring surface irregularities of crystals. Heights or optical paths as small as 10 Å have been measured. The method is capable of measuring the height of the mica molecule.

In the modification known as channel spectra,[3] the image of the object specimen is projected by the multiple beam interference microscope upon the entrance slit of a wave length spectrometer. If the microscope is illuminated with white light, bright lines appear in the focal plane of the spectrometer at those wave lengths for which the total optical path between the interferometer plates (in this case, the metalized slide and cover glass) is an integral number of half wave lengths. The method of channel spectra is a useful adjunct for measuring large optical path differences between particle and surround with particles that have appreciable edge thickness. The interpretation of the corresponding channel spectra, however, becomes complicated unless one can neglect the dispersion of the phase changes that occur upon reflection at the metalized surfaces of the slide and cover glass.

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[2] With Dyson's interferometer microscope, successive bright fringes are separated in optical path by one wave length. The bright fringes are much broader than those of the multiple beam interference microscope.

[3] It is known also as the method of fringes of equal chromatic order, abbreviated as the feco method.

The phase microscope is not well suited to quantitative determination of optical path differences but is convenient for ascertaining whether the optical path (and hence refractive index) of the particle is slightly higher or lower than that of the surround.

3. MEASUREMENT OF UNRESOLVED PARTICLES

A suitably modified polanret microscope is effective for measuring the area of unresolvably small opaque particles and for determining whether or not such particles are opaque. Areas corresponding to effective radii as low as 0.1 Airy unit[4] can be measured.

II. Principles of Phase Microscopy

1. A TYPICAL OPTICAL SYSTEM

A typical optical system for phase microscopy is illustrated in Fig. 1. It differs from that of the bright-field microscope only in the addition of a special condenser diaphragm *D* and a diffraction plate[5] *P*. A Kohler system of illumination is illustrated but critical or other forms of illumination are permissible. The essential requirement is that the condenser diaphragm shall be focused and centered upon the plane of the diffraction plate.

The opening in the condenser diaphragm is usually a narrow annulus centered on the optical axis but may assume other shapes[6] such as a slit or pinhole. The image of this opening in the plane of the diffraction plate describes the *conjugate area*. The remaining useful area of the diffraction plate is called the *complementary* area.

If the slide *S* contains no object specimen to diffract the incident beam of light, the entire beam passes undeviated through the slide and through the conjugate area. Some light is deviated from its course by diffraction at the object specimen. This deviated light is most likely to escape the conjugate area and to pass through the complementary area. The undeviated and deviated beams of light leaving the object specimen may therefore be said to pass through the conjugate and complementary areas, respectively, and to be separated at these two areas of the diffraction plate.

We shall see that brightness of the image is determined by interference[7]

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[4] An Airy unit is 0.61 λ/N.A. where λ denotes wave length and N.A. denotes numerical aperture of the polanret objective.

[5] Often called phase plate or Zernike disc.

[6] These additional shapes are useful for special applications.

[7] By the interference of two waves we mean their addition to form a single wave.

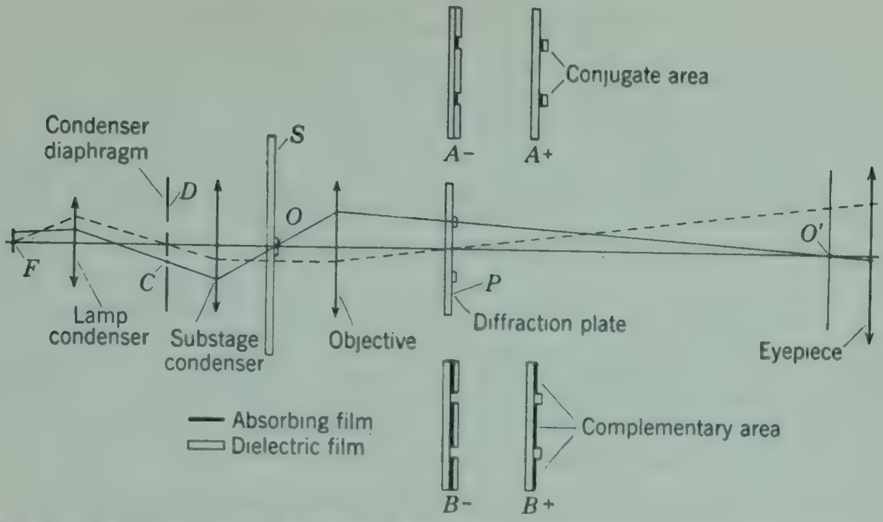

FIG. 1. A typical optical system for phase microscopy. Diffraction plates of the types $A-$ and $A+$ are the most useful. The diffraction plate falls within objectives that have short focal length.

of the undeviated and deviated waves upon their arrival in the plane of the image. The character of this interference, and hence contrast in the image, is determined by the relative strength (amplitude) and displacement (phase) of these two interfering waves. Because the undeviated and deviated waves are separated at the conjugate and complementary areas, their relative amplitude and phase can be controlled by coating the conjugate and complementary areas differently with absorbing and refracting (dielectric) materials.

The four varieties of diffraction plate are illustrated in Fig. 1. These are classified as the *A* or *B* type according as the absorbing material is added to the conjugate or complementary area. The *A* type is by far the more useful because the undeviated wave is ordinarily too strong as compared to the deviated wave. The letter of classification (*A* or *B*) is followed by a + or − sign to indicate that the refracting material has been placed so as to increase or to decrease, respectively, the optical path of the conjugate area relative to that of the complementary area. Let T denote the ratio of the light transmittance of the conjugate area to that of complementary area. Let δ denote the optical path difference in wave lengths between the conjugate and complementary area. $\delta > 0$ when the greater optical path belongs to the conjugate area. In the absence of a universally accepted convention, the notation $TA + \delta\lambda$ or $TB + \delta\lambda$ serves to indicate the most useful information about a diffraction plate. For example, $0.20\ A + \lambda/4$ indicates that light transmittance of the conjugate area is 20 per cent of that of the complementary area and that the optical path of the conjugate area exceeds that of the complementary area by $\lambda/4$. From the point of view of the simple vector theory of phase microscopy, it is only the parameters T and δ that matter. General theory and experiment show that the relative shape and dimensions of the conjugate and complementary areas affect the contrast.

The diffraction plate is usually a component of the microscope objective (the phase objective). Phase objectives now include practically all standard focal lengths and numerical apertures. They may be mounted upon a rotary nosepiece for rapid interchangeability. A separate condenser diaphragm is usually supplied for each phase objective and is mounted in a substage turret that facilitates their interchangeability. The diaphragm is omitted from one turret stop to permit bright field microscopy. With this open turret stop the iris of the substage condenser may be varied to control the numerical aperture of the illuminating beam. If such an iris is present, it should be left wide open whenever a phase diaphragm occupies the turret stop.

A separate short focus telescope replaces the eyepiece for viewing the plane of the diffraction plate while centering the image of the opening in the condenser diaphragm upon the conjugate area.

The condenser diaphragm is located at or near the first focal plane of the substage condenser. Correspondingly, the diffraction plate is located at or near the second focal plane of the phase objective. Accordingly, the object specimen is illuminated by parallel or nearly parallel bundles of light. Considerable departure from parallelism is tolerable.

In more complicated systems for phase microscopy, auxiliary lenses form successive images (Osterberg, 1948; Benford and Seidenberg, 1950) of the condenser diaphragm and the diffraction plate is placed at the most suitable of these image positions. Françon (Bennett *et al.*, 1951, p. 151) has devised a multipupil system in which the diffraction plate precedes the microscope objective.

2. ZERNIKE'S METHOD

Zernike's method is both the simplest and the most effective method of phase microscopy. The conjugate and complementary areas of the diffraction plate are coated differently but uniformly so that the light transmittance ratio T and the optical path difference δ of these areas are sensibly constant. The conjugate area is made so small that it intercepts an almost negligible amount of the deviated light waves.

One effect of spherical aberration of the objective is to spoil the constancy of δ. The design of the diffraction plate in influenced by spherical aberration. Since phase objectives are "corrected" for cover glasses having the standard thickness of 0.18 mm, cover glasses of this thickness should be used in order to avoid additional difficulties due to spherical aberration.

3. THE VECTOR THEORY OF PHASE MICROSCOPY

a. *Introduction*

Despite its many limitations,[8] the elementary vector theory of phase microscopy is useful because it is relatively simple and because it affords the first quantitative approximation to the laws of phase microscopy.

b. *Path of the Undeviated and Deviated Waves*

The discussion is simplified without altering the essential conclusions by choosing the opening in the condenser diaphragm as a pinhole on the optical axis. The conjugate area is then a small spot on the optical axis as in Fig. 2. We suppose that the source of light is focused upon the pinhole so that the pinhole acts as a secondary source. Let the pinhole be located at the first focal plane of the substage condenser. Coherent light from each point C in the pinhole emerges from the substage condenser as a plane wave front W. This wave front is practically parallel to the plane of the object specimen.

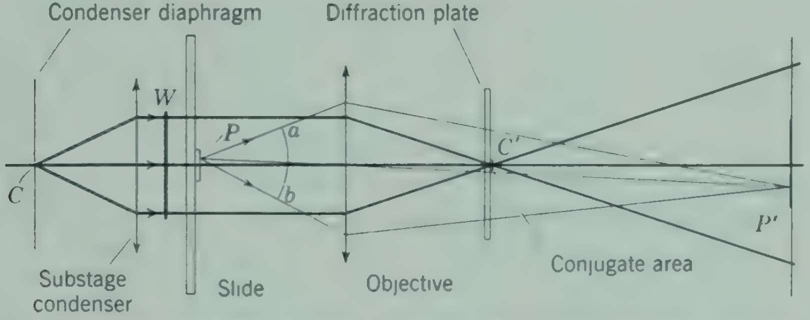

FIG. 2. An optical system in which the conjugate area is a small spot on the optical axis. The undeviated and deviated rays are drawn as heavy and light lines, respectively.

The undeviated rays are drawn as heavy lines in Fig. 2. After passing through the focus at C' in the conjugate area, the undeviated rays spread over the plane of the image. Therefore, the undeviated wave from each point of the secondary source passes through the conjugate area and spreads with fair degree of uniformity over the field of the image. The undeviated wave illuminates the entire image.

Deviated rays, drawn as light lines in Fig. 2, are bent from the incident beam by diffraction at the particle. A cone aPb of deviated rays originates at each point P in the neighborhood of the particle, spreads over the diffraction plate and comes to focus at the geometrical image of the particle. The deviated wave originates at the particle and illuminates the image of the particle. We note from Fig. 2 that most of the deviated wave passes through the complementary area provided that the conjugate area is small.

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[8] This theory does not predict, for example, the existence of the familiar halo around the image of the particle.

In summary, the image of the surround is illuminated by the undeviated wave alone. The image of the particle is illuminated by both the undeviated and the deviated waves. The undeviated waves pass through the conjugate area whereas most of the deviated waves pass through the complementary area of the diffraction plate.

c. *Basic Principles of Phase Microscopy*

Because the undeviated and deviated waves illuminate the image of the particle, the particle appears bright or dark according as these waves are made to interfere constructively or destructively in the image plane. Destructive interference will be complete and the particle will appear black when the conjugate and complementary areas of the diffraction plate are coated to equalize the amplitudes of the undeviated and deviated waves and to render these two waves out of phase (out of step) by one-half wave length.

We see how phase microscopy is accomplished by controlling the degree of constructive or destructive interference between the undeviated and deviated waves. The controlling elements consist of the diffraction plate and the associated condenser diaphragm.

d. *Vectorial Representation of Amplitude and Phase*

Amplitude and phase of light waves are represented as in Fig. 3 by the length $|V|$ of the vector V and by its phase angle θ, respectively. θ is positive in the counterclockwise direction of rotation from the vertical direction of reference v. Increasing θ corresponds to increasing optical path or phase. Optical path in number of wave length is converted to degrees by multiplication by 360. The illumination produced by the wave varies as the square of its amplitude.

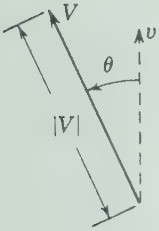

FIG. 3. Vectorial representation of amplitude and phase.

e. *The P and S-Waves*

The portion of the incident wave front W, Fig. 2, that passes through the particle is called the P-wave. If only one particle is present, the remaining portion of W passes through the surround. This portion is called the S-wave. More generally, that portion of W which passes through the surround becomes the S-wave. We regard the S-wave as the wave of reference. Therefore, we choose its phase angle as zero and draw the S-vector vertically with length unity.

Let Δ denote the optical path difference between the particle and an equal thickness of the surround. Δ is positive when the greater optical path

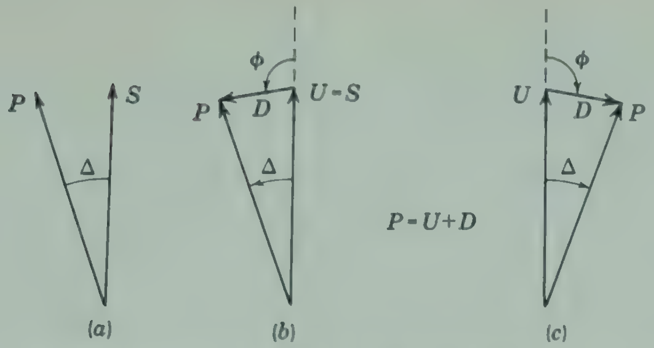

FIG. 4. Construction of vectors U and D from vectors P and S. The optical path difference Δ between particle and surround is positive and negative in Figs. 4B and 4C, respectively. Vectors P and S have the same length, as illustrated, when the particle and surround transmit alike.

belongs to the particle. It follows from the definition of the P- and S-waves that their phase difference is Δ. Hence the P-vector will be drawn counterclockwise or clockwise by the angle Δ from the S-vector according as Δ is positive or negative. The length of the P-vector is equal to the ratio of the *amplitude transmittance* of the particle to that of an equal thickness of the surround. Amplitude transmittance is equal to the square root of the usual light transmittance. The P- and S-vectors are equally long when particle and surround transmit equally, as is practically the case with most unstained biological particles.

If we suppose that the optical path of the particle exceeds the optical path of the surround by 20° ($\lambda/18$) and that particle and surround transmit equally, the P- and S-vectors will be drawn as in Fig. 4A. $\Delta = +20°$ and is counterclockwise.

f. The U and D-Waves

It is *always* possible physically and analytically to replace the P-vector, as in Fig. 4B, by two vectors U and D such that

$$P = U + D \tag{1}$$

and

$$U \equiv S. \tag{2}$$

By this simple act of vector construction a remarkable step has been taken; for the waves P and S can be replaced at will by the equivalent waves U and D. Note that the U-wave extends over the entire object plane. It is, in fact, the undeviated wave. Note that the D-wave vanishes when $P = U = S$, i.e. when the particle is indistinguishable from the surround. The D-wave is, in fact, the deviated wave.

g. Bright-field Microscopy

The idealized, bright-field microscope transports the U- and D-waves without further *relative* changes of phase and amplitude to the sharply focused image plane.

We have seen how the undeviated wave illuminates the image of the surround and how both the undeviated and deviated waves illuminate the image of the particle. Therefore, upon arrival in the image plane, the U- and D-waves produce, as illustrated in Fig. 5, the vectors S' and P' over the image of the surround and particle, respectively. The S'-wave and the P'-wave correspond to the S-wave and P-wave, respectively, of the object plane.

If the particle and surround transmit equally, $|P| = |S|$ and $|P'| = |S'|$. Consequently, the image plane is illuminated uniformly and the particle should be invisible in the sharply focused image plane, irrespective of the optical path difference Δ between particle and surround. Actually, particles are visible in the bright-field microscope when Δ is large enough because real objectives do not transmit the entire U- and D-waves without relative change to the image plane. Visibility is improved by one's tendency to select the most advantageous out-of-focus image plane. The effect of closing down the condenser diaphragm is to improve contrast by restricting even further the relative amount of deviated light that reaches the selected image plane.

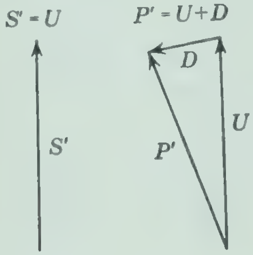

FIG. 5. Construction of the vectors S' and P' for the image of the surround and particle, respectively, in a bright-field microscope. The undeviated and deviated waves are assumed transported without relative change to the image plane.

One advantage of phase microscopy is the delineation of the particle in best contrast in the sharply focused image plane.

h. Phase Microscopy with $\Delta > 0$

Biological particles usually exceed their surround in refractive index. Correspondingly $\Delta > 0$ and P is drawn counterclockwise from S as in Figs 4A and B. Unstained biological particles transmit practically the same amount of light as their surround. Consequently for such particles we draw the P- and S-vectors of the same length.

The phase angle ϕ of the constructed D-vector, Fig. 4B, is slightly more

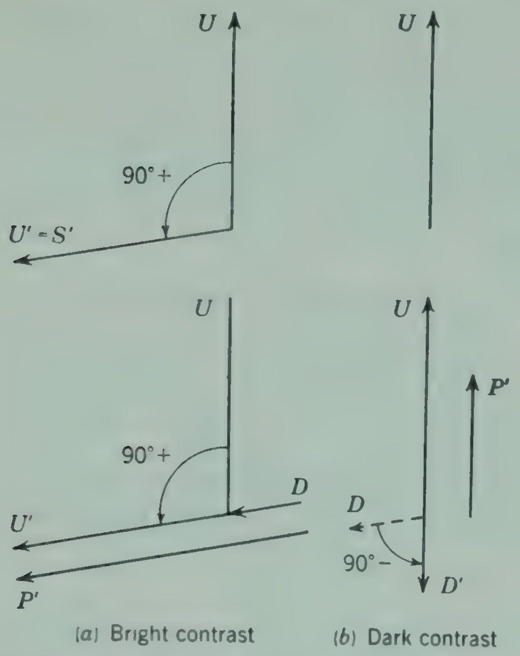

FIG. 6. Construction of the vectors S' and P' for the image of the surround and particle, respectively, in a phase microscope. The illustration applies to the case $\Delta = +20°$.

than $+90°$. Hence we see in a simple manner that when the optical path difference Δ is slightly greater than zero, the deviated wave exceeds the undeviated wave in phase by almost 90°, i.e., by almost $\lambda/4$.

Let the U-vector be rotated by the amount ϕ into parallelism with the D-vector as in Fig. 6A. This can be accomplished by increasing the optical path of the conjugate area by about $\lambda/4$ with respect to that of the complementary area. The U- and D-waves now arrive in phase in the sharply focused image plane and interfere constructively to produce the vector $P' = U' + D$ and the vector $S' = U'$ such that $|P'| > |S'|$ (P' longer than S'). Accordingly, the particle appears brighter than its surround.

On the other hand, let the D-vector be rotated by $180 - \phi$ degrees in the counterclockwise direction into antiparallelism with the U-vector as in Fig. 6B. This can be accomplished by increasing the optical path of the complementary area by almost $\lambda/4$ with respect to that of the conjugate area. The U- and D-waves now arrive out-of-phase and interfere destructively to produce a vector $P' = U + D'$ and a vector $S' = U$ such that $|P'| < |S'|$. Accordingly, the particle appears darker than its surround.

We note from the vector construction of Fig. 6B that $|P'| = 0$ so that the particle appears black when $|U| = |D'|$, i.e., when the amplitudes of the undeviated and deviated waves are equal. Since the amplitude of the undeviated wave usually exceeds that of the deviated wave, these amplitudes are ordinarily equalized by depositing the absorbing material upon the conjugate area of the diffraction plate as in the A type diffraction plate.

In summary, unstained biological particles for which the optical path difference Δ is slightly greater than zero will appear dark or bright[9] according as the diffraction plate is of the type $A - \lambda/4$ or $A + \lambda/4$.

i. Phase Microscopy with $\Delta < 0$

Let the optical path of the surround exceed slightly that of the unstained particle. Since $\Delta < 0$, the phase angle Δ of the P-vector is drawn clockwise as in Fig. 4C. The phase angle ϕ of the constructed D-vector is clockwise. The phase of the undeviated wave exceeds that of the deviated wave by slightly more than $\lambda/4$.

If the optical path of the complementary area is increased by approximately $\lambda/4$ with respect to that of the conjugate area, the phases of the deviated and undeviated waves will be equalized to produce constructive interference. The particle will appear brighter than its surround.

TABLE I

Relation between sgn(Δ) and the Type of Diffraction Plate

Contrast	Diffraction plate	$sgn(\Delta)$
Bright	$A + \lambda/4$	$+$
Dark	$A - \lambda/4$	$+$
Bright	$A - \lambda/4$	$-$
Dark	$A + \lambda/4$	$-$

On the other hand if we increase the optical path of the conjugate area by the amount $\lambda/4$ with respect to the complementry area, the phase of the undeviated wave is made to exceed that of the deviated wave by approximately $\lambda/2$. The two waves now interfere destructively and thus cause the particle to appear darker than its surround. Again, destructive interference will be complete and the particle will appear black if we equalize the amplitudes of the undeviated and deviated waves by depositing a suitable amount of absorbing material upon the conjugate area of the diffraction plate.

In summary, unstained biological particles for which the optical path difference Δ is slightly less than zero appear dark or bright according as one chooses a diffraction plate of the type $A + \lambda/4$ or $A - \lambda/4$.

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[9] The term dark contrast or bright contrast will be used to describe the state of contrast according as the particle appears dark or bright. The terms positive contrast and negative contrast are sometimes used instead of dark contrast and bright contrast, respectively.

j. Determination of the Sign of Δ

Whenever the magnitude of the optical path difference Δ between unstained particles and the surround is small, the sign of Δ can be determined without ambiguity from the type of diffraction plate required for producing bright or dark contrast. The relation between the sign[10] of Δ denoted by $sgn(\Delta)$, and the type of diffraction plate is given by Table I.

III. Useful Quantitative Relations

1. INTRODUCTION

The following quantitative relations are derivable (Bennett *et al.*, 1951, pp. 37–48) from the vector theory of Section II, 3. These relations predict the relative brightness of particle and surround. The required properties of the diffraction plate for producing a desired degree of contrast can be computed if the optical properties of the specimen are given. Conversely, it is sometimes possible to deduce in a satisfactory manner the properties of a particle from a knowledge of the state of contrast and the properties of the diffraction plate.

2. BRIGHTNESS OF THE PARTICLE AND SURROUND

Let G denote illumination or brightness of the sharply focused image plane. G_p and G_s are proportional to the illumination of the image of the particle and surround, respectively. g is the ratio of the amplitude transmittance[11] of the particle to that of an equal thickness of its surround. Δ is the optical path difference in radians or degrees between the particle and an equal thickness of its surround. Δ is considered positive when the optical path of the particle exceeds that of its surround. h is the ratio of the amplitude transmittance of the conjugate area of the diffraction plate to that of complementary area. δ is the optical path difference in radians or degrees between the conjugate and complementary areas. δ is considered positive when the optical path of the conjugate area exceeds that of the complementary area. Let h_1 denote the amplitude transmittance of the complementary area. One may set $h_1 = 1$ without any essential loss of generality.

It can be shown that

$$G_s = h_1^2 h^2; \tag{3}$$

$$G_p = h_1^2[(h \cos \delta + g \cos \Delta - 1)^2 + (h \sin \delta + g \sin \Delta)^2]. \tag{4}$$

In summary, Eqs. 3 and 4 determine the illuminations G_s and G_p of the surround and particle, respectively, within the scope of the elementary theory of phase microscopy.

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[10] $sgn(\Delta)$ is positive when the refractive index of the particle exceeds that of its surround.

[11] Amplitude transmittance is equal numerically to the square root of the usual light transmittance.

The constrast K, defined by the relation,

$$K \equiv \frac{G_p - G_s}{G_s} \tag{5}$$

can be determined from Eqs. 3 and 4.

3. Conditions for Darkest Contrast

a. The Most General Condition

The particle is said to appear in dark or bright contrast according as the particle appears darker or brighter than its surround. At darkest contrast $G_p = 0$.

It follows directly from Eq. 4 that $G_p = 0$ when

$$h \cos \delta + g \cos \Delta - 1 = 0;$$

$$h \sin \delta + g \sin \Delta = 0. \tag{6}$$

These comprise a pair of simultaneous equations for determining h and δ from g and Δ, or vice versa. The solutions for h and δ are

$$h = (1 - 2g \cos \Delta + g^2)^{1/2}; \tag{7.1}$$

$$\sin \delta = \frac{-g \sin \Delta}{h}; \tag{7.2}$$

$$\cos \delta = \frac{1 - g \cos \Delta}{h}. \tag{7.3}$$

In summary, Eqs. 7 determine the optical properties h and δ of the diffraction plate for securing darkest contrast with a particle having the given optical properties g and Δ. h is the ratio of the amplitude transmittance of the conjugate area to that of the complementary area. δ is the optical path difference between these two areas of the diffraction plate. δ is positive when the optical path through the conjugate area exceeds that through the complementary area. g is the ratio of the amplitude transmittance of the particle to that of an equal thickness of the surround. Δ is the optical path difference between the particle and an equal thickness of the surround. Δ is positive when the greater optical path belongs to the particle.

b. Small Optical Path Differences Δ

The amplitude transmittances of unstained biological particles and their surround are so nearly alike that we may set

$$g = 1. \tag{8}$$

By introducing $g = 1$, we find that Eqs. 7 for darkest contrast reduce to the set

$$h = 2\left|\sin\frac{\Delta}{2}\right|; \tag{9.1}$$

$$\delta = \frac{\Delta}{2} - \frac{\pi}{2} \quad \text{if} \quad 0 < \Delta \leqq \pi; \tag{9.2}$$

$$\delta = \frac{\Delta}{2} + \frac{\pi}{2} \quad \text{if} \quad -\pi < \Delta < 0; \tag{9.3}$$

wherein $|\sin \Delta/2|$ denotes the absolute value of $\sin \Delta/2$ and Δ is expressed in radians. Eqs. 9 apply to unstained biological particles having any optical path difference Δ.

With many particles Δ is so small that one may set

$$\sin\frac{\Delta}{2} = \frac{\Delta}{2}. \tag{10.1}$$

By introducing Eq. 10.1 into Eqs. 9 and by neglecting $\Delta/2$ in comparison with $\pi/2$, we find that the conditions for darkest contrast are well approximated by

$$h = |\Delta|; \tag{10.2}$$

$$\delta = -\pi/2 \text{ if } \Delta > 0; \tag{10.3}$$

$$\delta = \pi/2 \text{ if } \Delta < 0. \tag{10.4}$$

Since $\pi/2$ radians are equivalent to $\lambda/4$, we see that for unstained particles having small optical path differences (so small that $\sin \Delta/2 \rightarrow \Delta/2$) darkest contrast is obtained with a $TA - \lambda/4$ or a $TA + \lambda/4$ diffraction plate according as $\Delta > 0$ or $\Delta < 0$. The value of T is given by

$$T = h^2 = \Delta^2 \tag{10.5}$$

with Δ expressed in radians.

Suppose, for example, that $\Delta = \lambda/18$ or 20°. Then $\sin \Delta/2 = 0.1737$ and $\Delta/2 = 0.1746$ radians. The criterion of smallness of Δ, $\sin \Delta/2 = \Delta/2$, is well satisfied. From Eq. 10.2, $h = 0.349$. From Eq. 10.5, $T = 0.122$. Hence darkest contrast is produced by an $0.122\ A - \lambda/4$ diffraction plate. This conclusion is in accord[12] with experiment.

c. *Weakly Absorbing Particles*

Contrast in the image of weakly absorbing particles can be improved (and even reversed) by the proper choice of diffraction plate.

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[12] T-values in the range 0.1 to 0.3 are commonly used in commercial phase objectives.

Let us consider the class of particles for which $\Delta = 0$ but $g \neq 1$. By introducing $\Delta = 0$ into Eqs. 7, we find that the conditions for darkest contrast become

$$h = |1 - g|; \tag{11.1}$$

$$\delta = 0 \text{ when } g < 1; \tag{11.2}$$

$$\delta = \pi \text{ when } g > 1. \tag{11.3}$$

If $g > 1$, the particle absorbs less than an equal thickness of its surround. As $g \rightarrow 1$, the amplitude transmittance of particle and surround become alike and the required h-value for darkest contrast approaches zero. The penalty for decreasing h is to decrease the brightness G_s of the surround, see Eq. 3, until finally the whole field appears black.

In summary, darkest contrast with particles that transmit slightly differently from their surround is produced by a $TA + 0\lambda$ or a $TA \pm 0.5\lambda$ diffraction plate according as $g < 1$ or $g > 1$. The required value of T is

$$T = (1 - g)^2. \tag{11.4}$$

4. Bright Contrast

a. *The Schlieren Case*

Schlieren microscopy is obtained by setting $h = 0$, i.e., by making the conjugate area of the diffraction plate opaque.

We see from Eqs. 3 and 4 that when $h = 0$

$$G_s = 0; \tag{12.1}$$

$$G_p = h_1^2(1 - 2g \cos \Delta + g^2). \tag{12.2}$$

Now, the quantity $1 - 2g \cos \Delta + g^2$ is not equal to zero unless $\Delta = 0$ and $g = 1$, i.e., unless the particle is indistinguishable from its surround. Hence it follows that $G_p \neq 0$ when $G_s = 0$. The schlieren case, $h = 0$, produces brightest contrast because the particle appears illuminated whereas the surround does not.

The effect of making the conjugate area opaque is to block the undeviated wave. Consequently, the image formed under schlieren conditions is deteriorated because it is formed by the deviated light alone.

b. *Optimum Bright Contrast*

We define optimum bright contrast as the brightest contrast that can be obtained by varying δ with h fixed at its value for darkest contrast. It can be shown (Bennett *et al.*, 1951, p. 56) from Eqs. 3 and 4 that if δ_d is the value of δ at darkest contrast, the value δ_b for optimum bright contrast is

$$\delta_b = \delta_d + \lambda/2. \tag{13}$$

This means that contrast is reversed from darkest contrast to optimum bright contrast by adding λ/2 to the optical path difference between the conjugate and complementary areas.

The amplitudes $|U|$ and $|D|$ of the undeviated and deviated waves upon arrival in the image plane are (see Bennett *et al.*, 1951, p. 54)

$$|U| = h_1 h; \tag{14.1}$$

$$|D| = h_1(1 - 2g\cos\Delta + g^2)^{/12}. \tag{14.2}$$

It follows directly from Eqs. 7.1 and 14 that at darkest or optimum bright contrast

$$|U| = |D| = h_1 h, \tag{15}$$

i.e., the amplitudes of the undeviated and deviated waves are equalized by the diffraction plate.

The exact values h, δ for darkest or optimum bright contrast with a given specimen are rarely met by an available diffraction plate. Fortunately, the parameters h, δ can depart considerably from these exact values without marked loss of contrast. For this reason, a single diffraction plate can be used successfully[13] with particles having a limited range of values g and Δ.

IV. Particles With Inclusions

With respect to an inclusion, the immediate surround is the particle. Let Δ_i denote the optical path difference between the inclusion and an equal thickness of the particle with $\Delta_i > 0$ when the greater optical path belongs to the inclusion. Let g_i denote the ratio of the amplitude transmittance of the inclusion to that of an equal thickness of the particle.

From the viewpoint of the elementary theory, it is natural to regard the particle as the true or effective surround with respect to the inclusion and to substitute Δ_i for Δ and g_i for g in applying the theory to the prediction of contrast. On this basis, if $|\Delta_i|$ is small and g_i is not far from unity, one should *always* expect the inclusion to appear in best contrast with an $A - \lambda/4$ or an $A + \lambda/4$ diffraction plate. This expectation is not fulfilled on the basis of the general theory of phase microscopy. Contrast between the inclusion and particle depends not only upon Δ_i and g_i but also upon Δ and g and, indeed, upon the relative dimensions of the inclusion and particle.

Unpublished and, as yet, scattered calculations have shown that B type diffraction plates are occasionally required for revealing the inclusion with best contrast and that this need arises most frequently when the optical path difference between particle and surround approaches λ/2. It has been found experimentally that inclusions sometimes appear in best contrast with *B* type diffraction plates.

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[13] Also for this reason, the use of white illumination is practical.

V. Use of the Phase Microscope

1. Adjustment

The manufacturers' instructions should be followed by the beginner.

Those who are familiar with the bright field microscope may rotate the substage turret to the stop that contains no phase diaphragm and first adjust the system as a bright field microscope for Kohler[14] illumination. Centering of the substage condenser should be included and the objective left focused upon a standard slide.

Phase microscopy requires, in addition, that the opening in the phase diaphragm shall be focused upon the conjugate area of the diffraction plate and that the image of this opening shall fall *within* the conjugate area.

We shall call the variable iris diaphragm of the substage condenser the condenser iris and shall distinguish it from the phase diaphragms that are mounted in the substage turret. The condenser iris should be opened wide in phase microscopy and closed only to assist in making some of the adjustments.

Introduce the desired phase objective and the corresponding phase diaphragm. Replace the ocular by the centering telescope and focus this telescope upon the conjugate area. With *A* type diffraction plates, the conjugate area is dark and is usually annular. The bright image of the opening in the phase diaphragm should automatically be in good[15] focus upon the conjugate area. If not, recheck the focus of the objective with respect to the slide and, if necessary, rerack[16] the substage condenser slightly.

The opening in the phase diaphragm may appear slightly decentered with respect to the conjugate area. According to the maker, centering may be achieved by rotating two knobs that displace laterally the unit consisting of the condenser iris and substage condenser. Other makers provide, with advantage to the user, an additional pair of knobs for centering the phase diaphragms independently in the turret.

When the centering adjustment is completed, the opening in the phase diaphragm should be seen projected within the conjugated area. If so, the phase microscope is ready for use. If not, the wrong phase diaphragm may have been selected, the objective may be defocused or the substage condenser may have been racked too far from its normal position. Some lack of focus of the phase diaphragm with respect to the conjugate area is inevitable but is tolerable so long as the image of the opening in the phase diaphragm does not extend beyond the conjugate area. Undue scattering by the specimen diffuses the image of the opening in the phase diaphragm and prohibits critical adjustment. In fact, specimens that scatter appreciably are not suitable for phase microscopy. Diatoms and other specimens that produce a great deal of deviated light by diffraction illuminate the complementary area unduly for the purposes of adjustment.

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[14] Consult a textbook on microscopy. In Kohler illumination the lamp filament is focused upon the iris of the substage condenser and the iris of the lamp is focused by the substage condenser upon the object specimen.

[15] This focus will lack sharpness on account of the aberrations of the system of lenses between the phase diaphragm and the diffraction plate.

[16] If the phase diaphragm has not been located at the first focal plane of the substage condenser, the position of the substage condenser affects the sharpness of focus of the phase-diaphragm upon the diffraction plate.

Before replacing the ocular, one may check the centering of the substage condenser by closing down the condenser iris almost to the outer edge of the conjugate area. The slightly blurred image of the iris should be concentric with the conjugate area.

2. PREPARATION OF THE SPECIMEN

Isolated, colorless, isotropic particles located in a plane and immersed in a homogeneous medium form an ideal specimen for phase microscopy. The presence of extra particles arranged in depth and crowded laterally on the slide is to complicate enormously and unfavorably the system of deviated and undeviated waves that leave the object specimen. Deterioration of contrast can be expected as the penalty. Preparations containing particles in suspension should therefore be quite dilute. Tissue samples should be made sufficiently thin. Larger specimens are preferably reduced by such means as crushing, teasing, and sedimentation.

The cover glass should be of standard thickness (0.18 mm) to avoid loss of contrast from additional spherical aberration.

Cover glasses and slides having excessive striae, wedge, or waviness should be discarded. The effect of wedge may be to decenter the image of the opening in the phase diaphragm with respect to the conjugate area.

Whenever a choice of refractive index of the immersion medium exists, one can select the immersion medium so as to obtain either bright or dark contrast with a given diffraction plate. Even the degree of contrast can be controlled through the choice of difference of refractive index between the immersion medium and the particles.

The surface (and often details below it) of opaque specimens may be observed with the phase vertical illuminator provided that the surface can be prepared so as to reflect specularly. Other opaque specimens can be studied by making a replica of the surface and by examining this replica (Scott and Wyckoff, 1949; Wyckoff, 1947).

Preparation of the specimen has been discussed in much greater detail by O. W. Richards (Bennett *et al.*, 1951, pp. 172–184).

VI. Systems for Variable Phase Microscopy

1. PURPOSE

The variable phase microscope allows continuous variation of either or both of the chief parameters h and δ. h is the ratio of the amplitude transmittance of the conjugate area to that of the complementary area. δ is the optical path difference between these two areas of the diffraction plate. The variable phase microscope reveals a greater variety of specimens in excellent contrast. However, the greatest ultimate value of the variable phase microscope may well depend upon its use as a measuring instrument whereby certain properties of the specimen are deduced from the settings of h and δ.

2. POLANRET METHODS

The polanret method for variable phase microscopy involves replacing the diffraction plate by one of many possible combinations of polarizing, analyzing and retarding elements. Such combinations have been described by Osterberg (1947, 1946), Taylor (1947), Hartley (1947), Kastler and Montarnal (1948) and Locquin (1948). A related method that takes advantage of polarization phenomena at Brewster's angle has been described by Françon and Nomarski (1950a, 1950b).

Polanret methods become relatively simple both in principle and in execution when it suffices to vary either δ only or h only. Polanret methods are more easily achieved with objectives of long focal length.

The point of departure in one class of polanret systems is to polarize the undeviated and deviated waves linearly along different directions of vibration. These directions are preferably orthogonal. This state of polarization can be obtained by covering the conjugate and complementary areas by separate zonal polarizers, Z, whose transmission directions E_1 for the conjugate area and E_2 for the complementary area are perpendicular as in Fig. 7. After passing through the zonal polarizers, the undeviated and deviated waves remain separated physically on account of their different states of polarization until components of the two polarized waves are brought into a common direction of vibration by passing these waves through an analyzer. The phase difference, δ, and the amplitude ratio, h, can now be varied by the interaction of other polarizing and birefringent elements that do not have to be adjacent to the conjugate and complementary areas.

Experience indicates that h is preferably chosen as variable if one must fix either h or δ. The system of Fig. 7 permits continuous variation of the amplitude ratio, h, at two values of δ that differ by one-half wave length. Let the transmission directions of the polarizer, P, and of the analyzer, A,

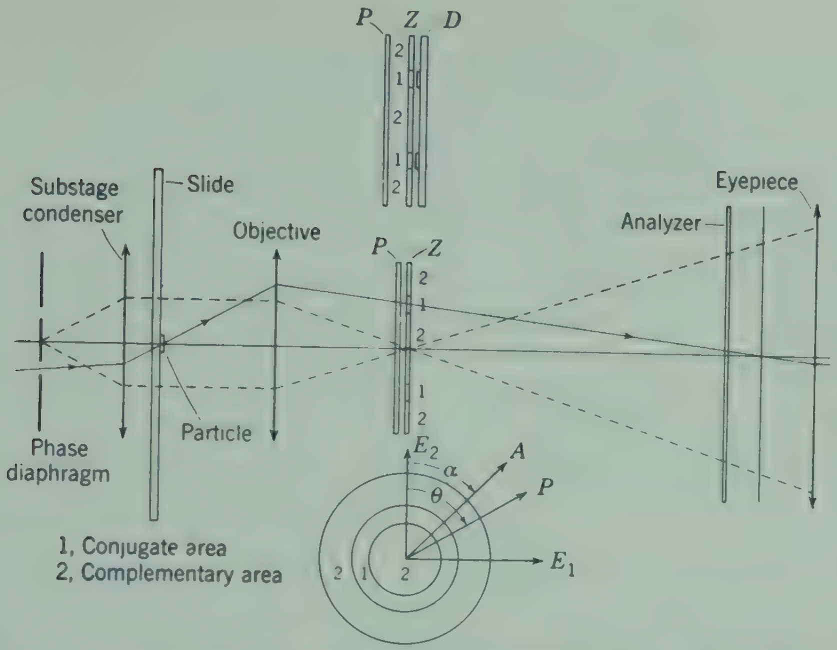

FIG. 7. A polanret system for achieving variable phase microscopy. The amplitude-transmittance ratio of the conjugate and complementary areas is varied continuously by rotation of the analyzer. The optical path difference δ jumps discontinuously by the amount $\lambda/2$ as the analyzer A crosses the directions of polarization E_1 and E_2 of the conjugate and complementary areas, respectively.

make the angles θ and α, respectively, with the transmission direction, E_2, of the complementary area. Then

$$h^2 = T = \tan^2 \theta \tan^2 \alpha \tag{16}$$

provided that E_1 and E_2 are perpendicular and that all of the polarizers are "perfect." It is convenient to fix the polarizer, P, at the orientation $\theta = 45°$. Rotation of the analyzer now varies h according to the law

$$h^2 = \tan^2 \alpha. \tag{17}$$

Polanret systems exemplified by Fig. 7 have the important property that the phase difference δ jumps discontinuously by $\lambda/2$ as the analyzer is rotated across the direction E_1 or E_2. The "neutral quadrant" contains the transmission direction P of the polarizer. It falls between E_1 and E_2 in Fig. 7. Opposite quadrants are equivalent. When the transmission direction A of the analyzer falls in the neutral quadrant, δ is equal numerically to the optical path difference between the conjugate and complementary areas. For example, if this path difference is zero, δ is zero or one-half wave length according as A falls within or outside the neutral quadrant. Suppose that the optical path difference between the conjugate and complementary areas is made $+\lambda/4$. This can be done by thickening the zonal polarizer on the conjugate area or by adding a diffraction plate D as in Fig. 7. Then $\delta = +\lambda/4$ or $\delta = -\lambda/4$ according as the transmittance direction A falls inside or outside the neutral quadrant. Therefore, sharp contrast reversals occur as A passes into or out of the neutral quadrant.

The uniform polarizer[17] P, Fig. 7, may be located anywhere between the source and the zonal polarizers Z. The presence of lenses between P and Z spoils the linearity of the polarized light incident upon the zonal polarizers. If P is located below the stage, the object is illuminated in polarized light.

If the optical path difference between the conjugate and complementary areas is not zero, this optical path difference, and hence δ, will vary continuously with wave length. A limited, controlled and continuous variation in δ is therefore possible with the system of Fig. 7 if one is willing to accept the expedient of changing the wave length (or a narrow band of wave lengths) continuously. The range of variation of δ can be increased, for example, by increasing the optical path difference at a wave length λ_0 from one-quarter to three-quarter wave lengths. The wave length can be varied inexpensively with minimum loss of light by rotating a high transmission, narrow band-pass, interference filter located between the illuminator and the phase diaphragm.

3. Interferential Diffraction Plates

Dufour and Lacquin (1951) have described and developed a variable phase microscope[18] wherein the diffraction plate is a modified interference filter of the narrow band-pass type. The conjugate area is coated by two transparent metallic coatings spaced by a dielectric such as magnesium fluoride, as illustrated in Fig. 8. This spacing governs the wave length λ_0 at which h^2 is maximum, Fig. 9. The values of h^2_{max} and h^2_{min} depend upon the choice of metal and thickness of the metallic coatings. The amplitude ratio, h, is varied between h_{min} and h_{max} by varying the wave length of illumination.

The interferential diffraction plate has the following important property. If the phase difference δ is equal to δ_1 at a wave length $\lambda_1 < \lambda_0$, then δ approaches $\delta_1 \pm \lambda/2$ at a wave length $\lambda_2 > \lambda_0$ where λ_1 and λ_2 are sufficiently different from λ_0 as illustrated in Fig. 9. The values $\delta_1 \pm \lambda/2$ are indistinguishable physically. Contrast reversal is thus possible by changing the wave length from λ_1 to the neighborhood of λ_2.

Whenever necessary, a second dielectric coating d, Fig. 8B, can be added

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[17] Polarizer P can be omitted when one does not need the neutral quadrant. The transmittance ratio $T = h^2$ then obeys Eq. 17 but the phase difference δ cannot be changed by 180°.

[18] Manufactured by Wild.

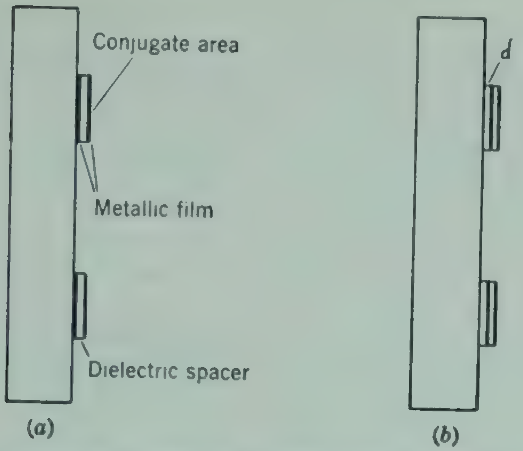

FIG. 8. Dufour and Locquin's interferential diffraction plate.

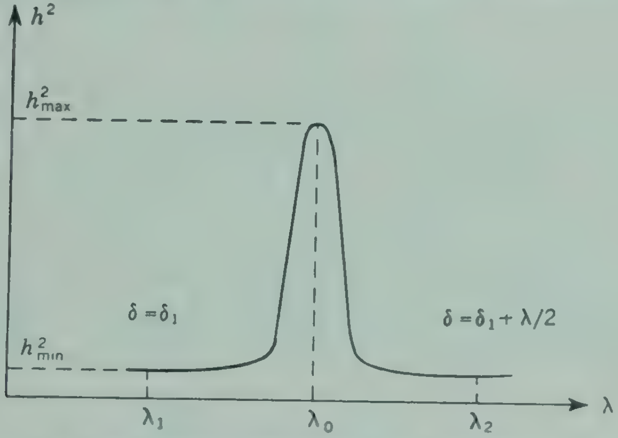

FIG. 9. The narrow band-pass properties of the interferential diffraction plate.

for gaining additional control of δ_1. If $\delta_1 = \lambda/4$, the phase difference δ between the undeviated and deviated waves can be varied continuously from $\lambda/4$ to $-\lambda/4$ by changing the wave length from λ_1 to λ_2.

In principle, the necessity of changing the wave length can be avoided by arranging to tilt the diffraction plate with respect to the optical axis. However, this procedure introduces several optical and mechanical difficulties.

The microscope may be illuminated by placing rotatable, narrow or wide pass band, interference filters between the source of light and the phase diaphragm. Rotation of wide pass-band interference filters produces a form of variable color contrast called Wild-Varicolor.

4. WEDGED CONJUGATE AREAS

It is possible to vary h and δ continuously by coating the conjugate area with "wedged" distributions of refracting and absorbing materials. Variability of h and δ is attained by relative motion of the coatings or by relative motion of a compound opening in the phase diaphragm with respect to the coatings. These methods are difficult to execute so as to preserve a high degree of axial symmetry of the optical system or so as to prevent an excessive amount of deviated light from passing through "unused" portions of the conjugate area.

VII. Measurement of Small Opaque Particles

The determination of the area, A, of unresolved opaque particles illustrates one of the possible uses of the variable phase microscope as a measuring instrument (Osterberg and Pride, 1950; Osterberg, 1952).

A sharply focused, opaque, and unresolvably small particle is seen as a diffraction image consisting of a central dark disk surrounded by diffraction rings. The center of the central disk is called the diffraction head. The diffraction image varies symmetrically above and below the position of sharpest focus provided that spherical aberration is negligible and provided that the particle is located near the optical axis.

The particle is illuminated by a monochromatic beam of narrow-coned axial illumination as in Fig. 10. The observer focuses the particle sharply, fixes his attention upon the diffraction head and varies the phase parameters h and δ until the diffraction head is darkest. If the particle is actually opaque, the phase difference δ will be zero. The area, A, in squared wave lengths is related to the amplitude ratio h of the polanret dark setting by the simple equation

$$A = \pi r_0^2 = h/\pi(\text{N.A.})^2 \tag{18.1}$$

where N.A. and r_0 denote the numerical aperture of the microscope objective and the effective radius of the particle, respectively.

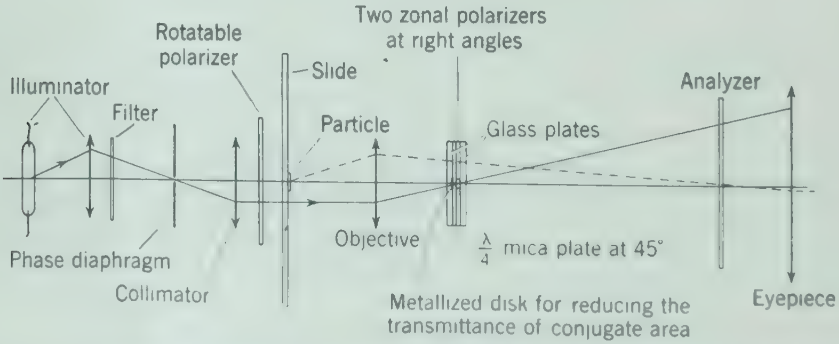
Rotatable polarizer
Two zonal polarizers at right angles
Slide
Illuminator
Analyzer
Filter
Particle
Glass plates
Phase diaphragm
Objective
$\frac{\lambda}{4}$ mica plate at 45°
Collimator
Metallized disk for reducing the transmittance of conjugate area
Eyepiece

FIG. 10. A polanret system for measuring the area of unresolvably small opaque particles. The phase parameters h and δ are varied continuously by rotating the analyzer and polarizer, respectively.

If R_0 denotes r_0 measured in Airy units where one Airy unit $\equiv$ 0.61 $\lambda/(\text{N.A.})$,

$$R_0 = h^{1/2}/1.92. \tag{18.2}$$

Radii of ½ Airy unit are at the limit of resolution of the bright field microscope. The corresponding h setting is almost unity. Because such h settings can be made with certainty, this method for measuring the effective radii of small opaque particles begins where the bright field microscope ends.

The polanret system of Fig. 10 allows h and δ to be varied independently. h is varied, as in the system of Fig. 7, by rotating the analyzer. The direction X of electrical vibration of the slow ray of the quarter-wave mica plate is fixed at 45° with respect to the transmission directions of the two perpendicular zonal polarizers. If θ is the direction between X and the transmission direction of the rotatable polarizer,

$$\delta = 2\theta. \tag{19}$$

In the system of Fig. 10, the conjugate area is a small disk centered on the optical axis. We see that conjugate areas of annular shape are not always superior for the many applications of phase microscopy.

VIII. The Phase Vertical Illumination Microscope

A simple system for phase microscopy with vertical illumination is illustrated in Fig. 11 (see Jupnik *et al.*, 1946, 1948; Cuckow, 1947; and Taylor, 1949). The condenser diaphragm is focused by the field lens and

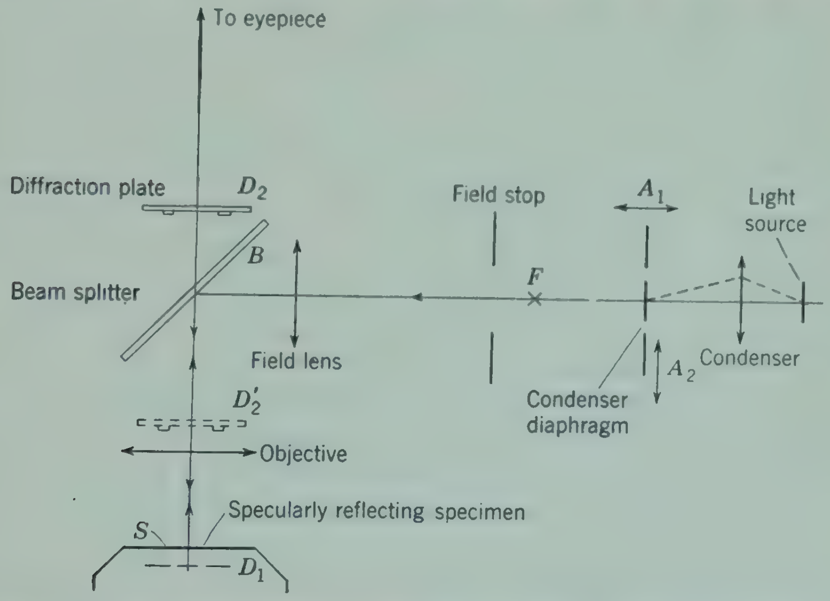
To eyepiece
Diffraction plate
D_2
Field stop
A_1
Light source
B
Beam splitter
F
Field lens
A_2
Condenser
Condenser diaphragm
D_2'
Objective
Specularly reflecting specimen
S
D_1

FIG. 11. The optical system of the simpler phase vertical illuminators.

the objective to form an image D_1 below the surface S. The diffraction plate is placed at the real image D_2 formed of D_1 above the beam splitter by the objective. The field stop is located inside the focal point F of the field lens and is focused upon S. The condenser diaphragm acts also as the aperture stop.

Alternately, the image D_2 may be formed at a position D_2' below the beam splitter. In this case the light passes through the diffraction plate twice. Stray light and losses of illumination are minimized by locating the diffraction plate above the beam splitter.

The system may first be adjusted as the usual conventional metallographic microscope. Additional care must be taken to orient the surface S perpendicular to the optical axis because the lateral location of D_2 depends upon the orientation of S. The objective is left focused upon the specimen. The centering telescope may now be inserted and focused upon the conjugate area of the diffraction plate. If means for making the adjustments A_1 and A_2 are provided, these means are utilized to focus and center the opening in the condenser diaphragm D upon the conjugate area of the diffraction plate. The annular opening in the condenser diaphragm should be projected *within* the conjugate area.

Benford and Seidenberg (1950) employ auxiliary lenses[19] to project an image of D_2 into a position more accessible for locating the diffraction plate.

The phase vertical illumination microscope is effective for revealing very shallow steps (etched or otherwise) in any surface S that possesses adequate specular reflectance. Elevations are distinguishable with certainty from the valleys provided that the optical path difference Δ presented by them does not exceed appreciably the amount for which $\sin \Delta \rightarrow \Delta$ with Δ expressed in radians. Provided that the phase changes produced by reflection are negligible or are sufficiently uniform, the optical path difference Δ is greater or less than zero according as the area under observation is a depression or an elevation. With an $A - \lambda/4$ diffraction plate, for example, the microscopically small area will appear darker or brighter than its surround according as the area is a depression or an elevation.

Adjacent areas may appear in different contrast because they differ slightly in the phase changes produced upon reflection from these areas. Such areas may lie below the surface if the surface is transparent.

It is worth noting that for revealing particles that have exceedingly small optical path differences with respect to their surround, the phase vertical illumination microscope is twice[20] as sensitive as the biological phase micro-

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[19] This system has been adopted by the Bausch and Lomb Optical Co. for use with their reflected light microscope.

[20] Since the light must pass through the slide twice, the optical path differences are doubled.

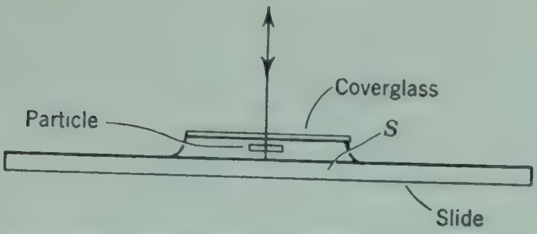

FIG. 12. Slide for viewing biological and other transparent particles by phase vertical illumination. The upper surface S of the slide is made highly reflecting.

scope. To take advantage of this fact, one may place the particle, Fig. 12, upon a slide having adequate surface reflectance S. The reflectance may be increased by nontoxic metallic coatings, e.g., inconel, or by dielectric coatings of high refractive index, e.g., titanium dioxide.

IX. Phase Color Contrast

The principles of phase microscopy can be utilized in many ways to obtain color contrast.

Zernike's method for color contrast is both direct and effective. The dispersions and thicknesses of the materials forming the conjugate and complementary areas are chosen so that poor phase cntrast occurs at one portion of the visible spectrum whereas good contrast occurs elsewhere in the visible spectrum.

In simple color phase, the optical path difference δ between the conjugate and complementary areas is made $\pm \lambda/4$ near one end and zero near the other end of the visible spectrum. If, for example, $\delta = +\lambda/4$ at the red end of the spectrum, particles appear red when their optical paths exceed that of the surround.

In reversible color contrast, δ is $+\lambda/4$ at one end, $-\lambda/4$ at the other end, and zero at the middle of the visible spectrum. Suppose, for example, that δ is $+\lambda/4$ at the red end of the spectrum. If the particle has slightly greater optical path than its surround, it appears red against a relatively neutral background when illuminated by white light, bright red against a darker red background when illuminated by red light and dark against a green background when illuminated by green light.

In a scheme[21] discussed by Grigg (1950), the opening in the Zernike condenser diaphragm for phase microscopy is covered by a filter that transmits color A and the opaque section of the condenser diaphragm is replaced by a second filter that transmits a contrasting color B of reducible intensity. With this arrangement, the significance of the conjugate and complementary areas as defined for the Zernike system of phase microscopy is of course modified. In the absence of an object specimen to produce a deviated wave, the light of colors A and B pass through the "conjugate" and "complementary" areas, respectively, of the diffraction plate. If, for example, δ is $+\lambda/4$ for color A and the optical path of the particle exceeds slightly that of its surround, the particle will appear in color A against a background of color intermediate between A and B.

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[21] More complete discussions of the methods of Zernike, Grigg and Barer has been given by H. Jupnik (Bennett et al, 1951).

Dufour and Locquin's method, Wild-Varicolor, is available commercially. The principles of this system have been discussed in Section VI, 3.

X. Interference Microscopy

1. DYSON'S METHOD

a. *Advantages of the Method*

The interferometer microscope described by Dyson (1950) reveals particles in enhanced contrast without introducing the halo[22] that borders the images of phase microscopy. Areas of large or small plate-like particles appear uniformly illuminated. Optical path differences, Δ, belonging to such particles are measured easily with adequate precision for biological research. Images of nonuniformly thick particles display interference fringes provided that the variation in the optical path through the particle exceeds one wave length. These fringes may be counted to determine the optical path difference approximately. Interpolation methods are available for measuring the fractional part that Δ exceeds the integral number of wave lengths determined by counting fringes.

The specimen is prepared on an ordinary slide and cover plate and is not exposed to metallic coatings as in the multiple beam interference microscope. Furthermore, the specimen is illuminated under higher numerical aperture than is feasible with a multiple beam interference microscope. On the other hand, the optical system is more complex than that of the multiple beam interference microscope or of the nonvariable phase microscope. More optical elements are present to impair image quality and to place additional demands upon the skill of the observer.

b. *The Optical System*

Dyson introduces an auxiliary optical system between the objective and the substage condenser as illustrated schematically in Fig. 13. The ray diagram indicates how the object specimen O and the fully silvered spot F are focused at the vertex V of the spherical reflector R whose spherical surface is fully silvered except for a small opening at V. The real image formed at V is re-imaged by the microscope objective at the eyepiece. The purpose of the reflector is, in effect, to increase the working distance of the

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[22] This is true also for the multiple beam interference microscope.

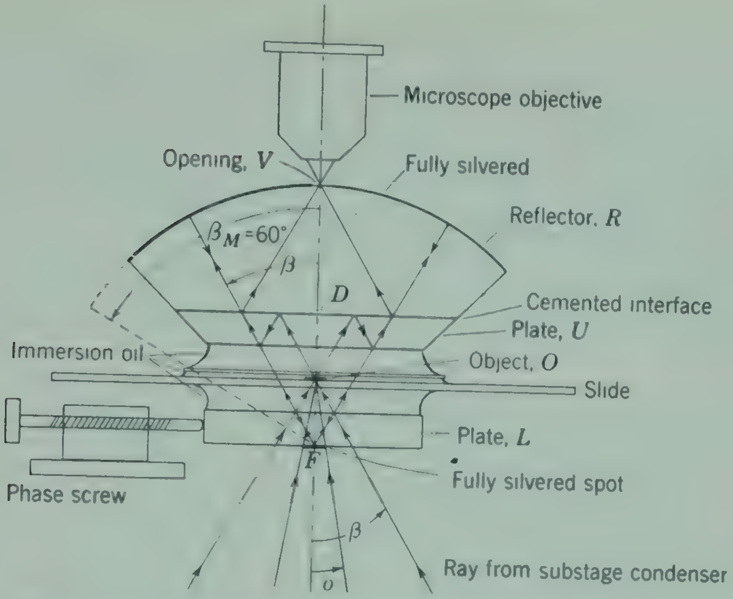
Microscope objective
Opening, V
Fully silvered
Reflector, R
β_M = 60°
β
D
Cemented interface
Plate, U
Immersion oil
Object, O
Slide
Plate, L
F
Fully silvered spot
Phase screw
β
o
Ray from substage condenser

FIG. 13. The auxiliary reflecting system of Dyson's interferometer microscope. The object O and fully silvered spot F are focused together with unit magnification at the unsilvered opening V. Both surfaces of the interferometer plate U and the upper surface of the interferometer plate L are half-silvered.

microscope objective for viewing the specimen through the relatively thick plate U. The interferometer consists of two similar, slightly wedged plates U and L.

The object slide is held in a mechanical stage between plates U and L. The gaps between the object slide and these plates contain immersion oil having refractive index similar to that of the microscope slide, the plates U and L and of the glass in reflector R.

Both major surfaces of interferometer plate U together with the upper surface of the lower plate L are half-silvered. The lower surface of L bears the fully silvered spot F whose area exceeds slightly the field of view. Plate U is cemented to the reflector R. The combination of the reflector R and plate U is ordinarily mounted upon the microscope objective and is spaced relative to the microscope objective so that the unsilvered opening at the vertex V (and hence the specimen together with the fully silvered spot F) is focused at the plane of the eyepiece.

Plates U and L are designed in relation to reflector R such that distances VD and DF are practically alike when the microscope is racked into focus upon the specimen. Furthermore, the spot F is then located at or near the center of curvature of the reflector. Both specimen and spot F are imaged with unit magnification at the vertex V.

This auxiliary reflecting system is similar in many respects to that adapted by Françon (Bennett *et al.*, 1951, pp. 151–155) for phase microscopy. As with Françon's system, the largest useful angle β_M of inclination which a ray can make with the optical axis is delimited to 60° (Bennett *et al.*, 1951, p. 153). Consequently, the largest numerical aperture, N.A., in Dyson's interference microscope is delimited to $n \sin 60°$ where n is the refractive index of the auxiliary system. If, for example $n = 1.52$, the maximum N.A. is 1.32. The object specimen is illuminated[23] by a hollow cone of light whose inner N.A. is $n \sin \alpha$ and whose outer N.A. is $n \sin \beta_{\max}$.

The microscope may be illuminated as in Kohler or critical[24] illumination. In either case, an illuminated field stop should be projected by the substage system upon the upper surface of the silvered spot F.

Turning now to the physical action of the interferometer, we note that the incident beam is split into two beams at the upper surface of plate L. One of these beams, the reference beam, is reflected toward F and thence toward V without passing through the specimen. The second beam, the *object beam*, passes through the object and thence toward the unsilvered opening at V. The reference and object beams arrive at V with equal strength, provided that the reflecting coatings have been chosen to the greatest advantage and provided that the object does not absorb light.

The optical path, and hence phase, of the object beam is modified by passage of the beam through the object. The optical path, and hence phase[25] δ, of the reference beam is varied by altering the thickness presented to the light beam by the slightly wedged plate L. Rotation of the phase screw displaces L along the direction of the wedge. The prismatic power of plate L is cancelled by that of the slightly wedged plate U.

At a particular setting of the phase screw, plates U and L present equal optical paths. This is the position of zero phase, $\delta = 0$. It is known also as the *white light position*, for at this position of the phase screw the field of a properly made and adjusted system will appear white under white illumination. At this position the field appears in maximum brightness irrespective of the wave length when monochromatic illumination is employed.

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[23] In drawing Fig. 13, it has been supposed that plate L and the substage condenser are also separated by immersion oil. Otherwise, the angles α and β refer to the rays after entry into plate L.

[24] Dyson chooses critical illumination, i.e., he focuses the source of illumination upon the specimen. He does not emphasize sharpness of this focus.

[25] The phase of the reference beam is modified also by particles located on the slide beyond the field of the fully silvered spot F, Fig. 13. We suppose that these particles are so few and so small that they have negligible effect upon the phase of the reference beam. The object slide should contain as few particles as possible when precise measurements are demanded.

c. Adjustment of the System

In a well adjusted system the illuminating beam is centered with respect to the optical axis, the wedges of plates U and L are in almost exact opposition and the planes of both plates are perpendicular to the optical axis.

The assembly consisting of reflector R and plate U is rotatable about the optical axis and mounted upon the microscope objective. Plate U is perpendicular to the optical axis. Levelling screws are provided in the substage system for adjusting plate L into parallelism with plate U. Alignment of the wedge directions of plates U and L is accomplished by rotating the reflector assembly.

The upper surface of the lower plate L, Fig. 13, is adjusted about 0.5 mm below the top surface of the stage. Immersion oil is dropped upon L and the object slide is brought into contact with the immersion oil. A second drop of oil is placed on top of the slide and the microscope tube is racked down until the lower surface of plate U is in contact with immersion oil. The field and aperture stops are closed down and the illuminating system centered. The field stop should appear centered and in good focus upon the plane of the object. However, two images of the field stop are usually visible. One brings these images into coincidence by tilting plate L with the aid of the leveling screws. The field and aperture stops are then opened.

With monochromatic illumination, evenly spaced interference fringes usually appear in the field of view. A second set of such fringes usually appear at the back aperture of the objective upon removal of the eyepiece. As the plane of plate L is brought into perpendicularity with the optical axis by means of the leveling screws and as the axes of the two wedged plates are rendered antiparallel by rotating the reflector R, the fringe width of both of these sets of fringes increases until the field of view and the back aperture of the objective appear quite uniformly illuminated. The width of the fringes seen at the back aperture of the objective is especially sensitive to the tilt of plate L.

Uniformly illuminated fields corresponding to infinite fringe width are usually the most advantageous for the purposes of observation.

Interference fringes reappear upon turning one of the leveling screws. These fringes move across the field of view as one rotates the phase screw, provided that the correct leveling screw has been disturbed. The fringes formed by white light are colored. The colors become more vivid as the white light position of the phase screw is approached. The central, bright, white fringe demarks the white light position with respect to these fringes of finite width. A small rotation of the phase screw moves the central white fringe to the center of the field of view. If the disturbed leveling screw is returned to its original setting, the field of view is uniformly illuminated at the white light position[26] of the phase screw. A minute rotation of the phase screw now locates the white light position of the phase screw more critically as judged from the over-all whiteness of the field. The setting of the phase screw for this white light position must be expected to vary slightly from one slide to another. If the slide contains a specimen, one ascertains the white light position by ignoring the specimen and adjusting the phase screw for most uniform whiteness of the background.

If an iris diaphragm has been provided in the substage system for controlling the numerical aperture of the incident beam, this diaphragm is preferably closed down, as in the conventional microscope, until the back aperture of the microscope objective is just filled[27] with light.

d. Theory and Interpretation

Let Δ denote the optical path difference between the particle and an equal thickness of its surround. $\Delta > 0$ when the greater optical path belongs to the particle. If the particle is nonuniform, Δ will vary. Let δ denote the change of optical path introduced into the reference beam by means of the phase screw. $\delta > 0$ when the optical path of the reference beam exceeds that of the object beam in the absence of the object. $\delta = 0$ at the white light position. δ is constant over the field of view provided that the microscope has been adjusted for a uniformly illuminated field of view.

We suppose that the dividing surfaces are half silvered such that the reference and object beams are equal in strength. Let E denote the brightness (proportional to the illumination) at any point in the image. Then

$$E = E_0 \cos^2 \frac{\delta - \Delta}{2} \tag{20}$$

where E_0 is the maximum value of E. Δ may be considered as zero for all points belonging to the background. At the white light position of the phase screw, $\delta = 0$ so that $E = E_0$ for all points in the background.

The quantity $\Delta - \delta$ is equal to the optical path difference[28] between the object beam and the reference beam. If $\Delta - \delta$ is an odd number ν of half wave lengths, i.e., $\nu\pi$ radians, we know that the reference and object beams interfere destructively to produce zero illumination. On the other hand, if $\Delta - \delta$ is any integral number m of wave lengths, i.e., $m2\pi$ radians, these beams interfere constructively to produce maximum illumination $E = E_0$.

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[26] It will be observed that white light positions of the phase screw could be defined for fringes of finite width but that these positions depend upon the location of the central white fringe within the field of view.

[27] In case the N.A. of the auxiliary reflecting system is less than that of the microscope objective, it will not be possible to fill the back aperture of the objective.

[28] Provided that the reference beam does not traverse too many particles beyond the field of the fully silvered spot.

However, we see directly from Eq. 20 that the illumination is zero at all points for which

$$\Delta - \delta = \nu\pi \text{ radians } (= \nu\lambda/2) \tag{21}$$

and that the illumination is maximum at points for which

$$\Delta - \delta = m2\pi \text{ radians } (= m\lambda). \tag{22}$$

The integers ν are odd but m is any integer or zero.

The locus of points for which the illumination is zero is customarily called a *dark fringe*. The locus of points having maximum illumination is called a *bright fringe*. If δ is set at the white light position, the fringes seen within the image of the particle are dark wherever

$$\Delta = \nu\pi \text{ radians } (= \nu\lambda/2);\ \nu \text{ odd}. \tag{21.1}$$

On the other hand, the fringes are bright wherever

$$\Delta = m2\pi \text{ radians } (= m\lambda);\ m = 0, 1, 2, 3, \text{ etc.} \tag{22.1}$$

We shall call ν and m *order numbers* when they are counted with respect to the white light position as in Eqs. 21.1 and 22.1. To each fringe belongs an order number. We see at once that the optical path differences Δ between particle and surround can be measured by determining order numbers. This determination is usually possible but is subject to many pitfalls in the hands of an unskilled observer. With particles having simple shape (for example a spherical particle), m is equal to the number of the bright fringe counted inward from the edge of the image of the particle. The determination of order numbers will be discussed in succeeding sections for particles having increasing degree of complexity.

The order numbers are negative when $\Delta < 0$.

It will be noted from Eqs. 21 and 22 that successive dark fringes or successive bright fringes are separated by one wave length (2π radians) in optical path. In the multiple beam interference microscope, Δ is increased only one-half wave length between successive bright fringes. This difference between Dyson's interferometer microscope and other interference microscopes is, if overlooked, a source of confusion.

e. Relative Motion of Fringes and the Phase Screw

Equation 22 states that the m^{th} bright monochromatic fringe must be located such that

$$\Delta - \delta = \text{constant} = m2\pi. \tag{22.2}$$

Suppose that δ is increased from the white light position, $\delta = 0$. Then Δ must be increased in order to satisfy Eq. 22.2. This means that the m^{th}

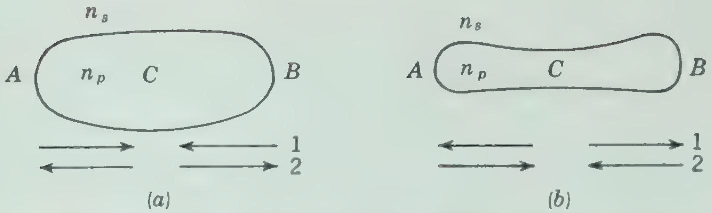

FIG. 14. Movement of the monochromatic fringes. Figures 14a and 14b refer to geometrically convex and geometrically concave particles, respectively.

bright fringe will move into a region of higher Δ. On the other hand, if δ is decreased by rotation of the phase screw, the fringes move into regions having lower Δ. In summary, *the monochromatic fringes move into regions within the particle having higher or lower optical path differences* Δ *with respect to the surround according as the phase screw is rotated so as to increase or decrease* δ. This statement is true whether Δ is positive or negative.

A geometrically convex particle is illustrated in Fig. 14a. If $n_p > n_s$, $\Delta > 0$ and the particle is optically convex. Δ increases toward C. Hence the fringes will move in the arrow direction 1 or 2 according as δ is increased or decreased. This particle is, however, optically concave when $\Delta < 0$; for then Δ decreases toward the center C. The fringes now move in the arrow direction 2 or 1 according as δ is increased or decreased. The particle illustrated in Fig. 14b is geometrically concave, but is optically concave or convex according as $n_p > n_s$ or as $n_p < n_s$. If $n_p > n_s$, the fringes move in the arrow direction 1 or 2 according as δ is increased or decreased. But if $n_p < n_s$, the fringes move in the arrow direction 2 or 1 according as δ is increased or decreased. We see that the relative motion of fringes and phase screw determines primarily *optical* convexity or concavity.

If the particle is known to be geometrically convex or concave, the motion of the monochromatic fringes reveals the sign of Δ, i.e., reveals whether $n_p > n_s$ or $n_p < n_s$. Thus, if the particle is known to be geometrically convex as in Fig. 14a, $n_p > n_s$ when the fringes move in the arrow direction 1 or 2 according as δ is increased or decreased; but $n_p < n_s$ when the fringes move in the arrow direction 2 or 1 according as δ is increased or decreased.

f. Motion of the Pseudo White Fringe

Let n_p, n_s and n_g denote refractive indices of the object particle, surround and glass components of the interferometer, respectively.

The phase difference $(\Delta - \delta)$ of Eq. 22 can be made zero for a given Δ by the proper choice of δ for a wave length in the yellow-green portion of the visible spectrum of white illumination. If the dispersions of n_g and of $(n_p - n_s)$ do not differ appreciably, $\Delta - \delta$ will remain almost zero for other portions of the visible spectrum. Hence there will exist a position of the phase screw for which a white-like fringe appears within that region of the particle for which $\Delta - \delta \rightarrow 0$. We shall call this fringe the *pseudo white fringe*; for the true white fringe is, strictly, a property of the background and is observed at the setting $\delta = 0$ of the phase screw. *The pseudo white fringe is made to appear within the image of the particle by rotating the phase screw so as to increase or decrease* δ *from* $\delta = 0$ *according as* $\Delta > 0$ *or as* $\Delta < 0$.

It is frequently advantageous to tilt the lower plate L, Fig. 13, to produce a band of straight fringes within the background. The central bright fringe becomes the pseudo white fringe as this band of fringes is moved into the image of the particle by rotating the phase screw. Of course, the fringes seen within the image of the particle will not remain straight unless the particle is plate-like.

We observe also that *the pseudo white fringe moves into regions having higher or lower optical path difference* Δ *with respect to the surround according as the phase screw is rotated so as to increase or to decrease* δ.

g. Approximate Measurements with Convex Particles

In the simplest case, the edge thickness of the particle vanishes as in Fig. 14a. $\Delta = 0$ at the edge of the particle and $|\Delta|$ increases toward the center.

Let the phase screw be set at $\delta = 0$. Then the optical path difference $|\Delta|$ is λ at the location of the first bright fringe within the edge of the image of the particle, 2λ at the location of the second bright fringe, 3λ at the location of the third bright fringe, etc. Similarly, $|\Delta| = \lambda/2$ at the location of the first dark fringe within the edge of the image, $3\lambda/2$ at the second dark fringe, $5\lambda/2$ at the third dark fringe, etc. Suppose that $|\Delta| = 5\lambda$ at C, Fig. 14a. The center C will appear bright and will be surrounded by four other bright fringes. Suppose that $|\Delta| = 5.75\lambda$ at C. The central portion will appear grey. The first fringe surrounding C will be dark. This dark fringe will be surrounded by five bright fringes. The center C appears dark when the maximum $|\Delta|$ is $\lambda/2$, $3\lambda/2$, $5\lambda/2$, etc.

If it is inconvenient to shift from monochromatic to white light, the sign of Δ can be determined by utilizing the principles of Section *e*.

h. Approximate Measurements with Nonuniformly Convex Particles

Let the phase screw be set at $\delta = 0$ and let the illumination be monochromatic. When the particle has the more complicated shape illustrated in Fig 15, caution must be exercised in determining Δ from the fringe count because the

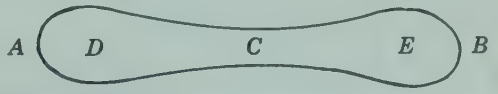

FIG. 15. A nonuniformly convex and concave particle.

fringe count is not necessarily equal to the order number m or ν. It is the order number that determines Δ.

Suppose for definiteness that $\Delta > 0$. Then m increases from B to E, reaches a maximum at E, decreases toward C and reaches a minimum at C. The fringes counted between B and C do not correspnd to order numbers.

Fortunately, the principles of either Section *e* or *f* enable one to determine whether the order number is increasing or decreasing in the direction of counting.

We know from Section *e* that the monochromatic fringes move toward regions of increasing Δ (and hence toward increasing order numbers m and ν whether these be positive or negative) as δ is increased from $\delta = 0$. If $\Delta > 0$, the fringes move toward E and D as δ is increased and move away from C. If $\Delta < 0$, the fringes surrounding C move toward C, but away from regions D and E as δ is increased. Since the motion of the fringes is in the direction of increasing order numbers[29] as δ is increased, the direction of increasing order numbers becomes known. (A negative order number increases in passing, for example, from -2 to -1.)

The interference microscope can be used to measure shape factors of particles that have constant refractive index. With reference to the particle illustrated in Fig. 15, suppose that Δ increases from 0 at B to 5λ at E and then decreases to 2λ at C. We deduce at once that the ratio of the thickness at C to that at E is 2/5. The thickness t of the particle can be computed from the measured value of Δ at any point within the particle. Thus

$$t = \frac{\Delta}{n_p - n_s} \lambda \tag{23}$$

when Δ is expressed[30] as number of wave lengths. The thickness t is in microns if, for example, λ is expressed in microns.

i. Calibration of the Phase Screw

In order to measure Δ for particles that have appreciable edge thickness, it is essential to calibrate the phase screw.

The simplest procedure is to employ monochromatic illumination of the preferred wave length λ and to determine the angle of rotation d_0 of the phase screw between the appearance of successive maxima or successive minima in the illumination of the background. The angle d_0 represents one wave length (2π radians) of variation in δ at the selected wave length. This calibration should be performed near the white light position.

Greater precision is attained by introducing a thin, uniformly thick particle (a plate-like particle). As the phase screw is advanced, the particle vanishes alternately against dark and bright backgrounds. The settings are sharp. If the phase difference Δ of the selected particle is a small fraction of one wave length, the sharper match will be that against the dark background. The angle of rotation, d_0, of the phase screw between successive dark matches or successive bright matches of the particle against background corresponds again to one wave length[31] (or 2π radians) variation in δ at the wave length of calibration. A flat-bottomed scratch in the glass surface bearing the fully silvered spot F, Fig. 13, will appear in focus in the field of view and may be utilized (see Dyson, 1950, p. 186) as the plate-like particle for the purposes of this calibration. This choice of particle is advantageous for circumventing back lash in the phase screw. Let the scratch be projected upon the scale of the eyepiece. As d_0, one may now employ the displacement of the projected scratch between successive like matches of the scratch against background.

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[29] The order numbers are positive when $\Delta > 0$, i.e., when δ has to be made greater than zero in order to bring the white fringe into the image of the particle.

[30] Thus expressed, Δ is equivalent to the dimensionless quantity of phase.

j. *Measurements with Plate-like Particles*

The plate-like particle illustrated in Fig. 16 is the simplest particle having nonvanishing edge thickness. The procedure of section g fails because one is unable to count the fringes at either of the edges A or B whenever the edges rise too abruptly. Furthermore, if Δ is less than ½ number of wave lengths,

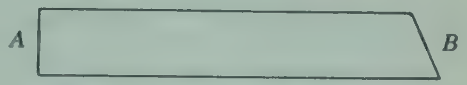

FIG. 16. A plate-like particle having a discontinuously abrupt edge at A and an abrupt edge at B.

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[31] The conditions for equal illumination of background and particle is seen from Eq. 20 to be

$$\cos^2 \frac{\delta}{2} = \cos^2 \frac{\delta - \Delta}{2}. \tag{20.1}$$

The solutions for δ are

$$\delta_m = \Delta + m2\pi \tag{20.2}$$

$$\delta_\nu = \Delta + \nu\pi \tag{20.3}$$

wherein $m = 0, 1, 2, 3$, etc. and $\nu = 1, 3, 5$, etc. Eqs. 20.2 and 20.3 correspond to the bright and dark matches, respectively, of the particle against its surround. Conversely

$$\Delta = \delta_m - m2\pi \tag{20.4}$$

or

$$\Delta = \delta_\nu - \nu\pi \tag{20.5}$$

where δ and Δ are expressed in radians.

We observe that this method enables one to measure Δ for plate-like particles when the calibration of the phase screw has been established.

no fringe count is possible because fluctuations in the illumination do not occur.

The following method applies to plate-like particles and to the plate-like portions of more complex particles. It is applicable to thick or thin particles. The method is not flawless but becomes highly precise as the dispersion of n_g approaches that of $n_p - n_s$.

The phase screw is first set at the white light position for the background and is next rotated through an angle d until the pseudo white fringe appears over the plate like portion of the particle. Then, simply,

$$\Delta = d/d_0 . \tag{24}$$

Δ is expressed as number of wave lengths at the wave length of calibration for d_0.[32] Δ is positive or negative according as the phase screw is rotated so as to increase or to decrease δ from the white light position, $\delta = 0$. The fraction d/d_0 may not be an integer. It approaches zero as $\Delta \rightarrow 0$.

k. *Convex Particles with Nonvanishing Edge Thickness*

A geometrically convex particle having nonvanishing edge thickness is illustrated in Fig. 17. Such particles are most flattopped in the central region C and are characterized by the fact that $|\Delta|$ is maximum in the region C.

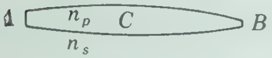

FIG. 17.

We set the phase screw at the white light position and determine the angle d of rotation of the phase screw to bring the pseudo white fringe into the region C. The optical path difference Δ is positive or negative according as d is positive[33] or negative. If $|d|$ assumes its maximum value $|d|_{\max}$ in the region C, the particle is geometrically convex. Furthermore, as in Eq. 24,

$$|\Delta|_{\max} = |\delta|_{\max} = |d|_{\max}/d_0 \tag{24.1}$$

wherein d_0 is the angle of rotation of the phase screw required to alter δ by one wave length at a specified wave length and Δ is expressed as number of said wave lengths.

We change from white to monochromatic illumination without disturbing the setting $|d|_{\max}$ of the phase screw. If the variation in Δ is large enough, several monochromatic fringes can be counted outward from the region C. The central fringe will be a bright spot provided that the phase screw has remained at the setting $|d|_{\max}$ and will be surrounded alternately by dark and bright fringes. We count the dark fringes outward from C as the *odd* numbers $\nu = 1, 3, 5$ etc. and count the bright fringes outward from C as the integers $m = 0, 1, 2, 3$, etc. with $m = 0$ for the central bright spot. Let $sgn(d)$ denote the sign of d, i.e., $sgn(d) \equiv d/|d| = \pm 1$. Then at the location of each dark fringe

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[32] The calibration constant d_0 is always positive.

[33] The positive direction d of rotation is that for which δ is positive.

$$\Delta = sgn(d)[-\nu/2 + |d|_{\max}/d_0] \qquad (21.2)$$

and at the location of each bright fringe

$$\Delta = sgn(d)[-m + |d|_{\max}/d_0]. \qquad (22.3)$$

A simpler procedure is used when the variation in Δ is small. One places the pseudo white fringe at any desired location within the image of the particle and applies Eq. 24.

1. Concave Particles with Nonvanishing Edge Thickness

A geometrically concave particle having nonvanishing edge thickness is illustrated in Fig. 18. Such particles are characterized by the fact that $|\Delta|$ is minimum in the region C.

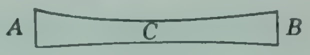

FIG. 18.

As with convex particles, we set the phase screw at the white light position and determine the angle d of rotation of the phase screw to bring the white fringe into region C. Δ is positive or negative according as d is positive or negative. If $|d|$ assumes its minimum value $|d|_{\min}$ in the region C, the particle is geometrically concave. Also,

$$|\Delta|_{\min} = |d|_{\min}/d_0 . \qquad (24.2)$$

With monochromatic illumination, the central fringe is again a bright spot provided that the phase screw is left (as it should be) at the setting $|d|_{\min}$. If the variation in Δ is large enough, we may count the dark fringes outward from the central bright spot as the odd integers $\nu = 1, 3, 5$, etc. and count the bright fringes outward from C as the integers $m = 0, 1, 2, 3$, etc. with $m = 0$ for the central bright fringe. At the location of each dark fringe

$$\Delta = sgn(d)\,[\nu/2 + |d|_{\min}/d_0]. \qquad (21.3)$$

At the location of each bright fringe

$$\Delta = sgn(d)\,[m + |d|_{\min}/d_0] \qquad (22.4)$$

wherein Δ is expressed as number of wave lengths at the wave length of calibration for d_0. If the variation in Δ is small, we may place the white fringe at any desired point within the image of the particle and apply Eq. 24. Both convex and concave particles may be measured as plate-like particles when the variation of $|\Delta|$ from $|\Delta|_{\max}$ or $|\Delta|_{\min}$ is so small that no fringe count is possible. When higher precision is desired, the method discussed in sub-section *o* may be applied.

m. Nonuniformly Convex and Concave Particles Having Nonvanishing Edge Thickness

A particle having nonvanishing edge thickness and having both convex and concave regions is illustrated in Fig. 19.

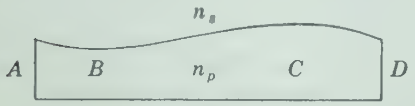

FIG. 19.

The values $|\Delta|_{\max}$ at C and $|\Delta|_{\min}$ at B can be determined with the aid of Eqs. 24.1 and 24.2 by moving the white fringe from the background into regions C and B. Provided that n_p is sensibly uniform, $|\Delta|_{\max}$ and $|\Delta|_{\min}$ must occur in regions having maximum and minimum thickness, respectively. Hence the thick portions of the particle can be distinguished from the thin portions. When n_p is constant, the ratio of the thickness at C to that at B is $|\Delta|_{\max}/|\Delta|_{\min}$. We know that Δ is positive or negative, i.e., $n_p > n_s$ or $n_p < n_s$, according as the phase screw has to be rotated so as to increase or to decrease δ from $\delta = 0$ in order to bring the white fringe from the background into the image of the particle.

The values of Δ in the neighborhood of $|\Delta|_{\max}$ and $|\Delta|_{\min}$ can be measured with the aid of Eqs. 21.2, 22.3, 21.3 and 22.4 by counting monochromatic fringes from C and B after the manner described in sub-sections *k* and *l*. If Δ varies slightly throughout the particle, one may apply the method of sub-section *j* by moving the pseudo white fringe over various portions of the image of the particle and by applying Eq. 24.

Geometrical convexity and concavity as well as optical convexity and concavity can be determined from the motion of the pseudo white fringe because the sign of Δ is established directly from the sign of the angle of rotation d of the phase screw.

n. Particles Having Inclusions

The principles and methods discussed in the above sections *d* to *m* apply to the measurement of the optical properties of suitably large inclusions. Let n_i denote the refractive index of the inclusion within a particle that has refractive index n_p. Then $n_i - n_p$ is greater or less than zero according as δ has to be increased or decreased in order to move the pseudo white fringe from the image of the particle into the image of the inclusion. If the inclusion is large enough and if n_i is sufficiently different from n_p, the variation in the optical path throughout the inclusion can be determined by counting monochromatic fringes within the image of the inclusion. If the variation contributed to Δ by the particle is sensibly zero over the area of the inclusion, the observed variation in Δ is contributed by the inclusion. In fact,

$$\Delta = \Delta_p + \Delta_i \tag{25}$$

wherein

$$\Delta_p \equiv (n_p - n_s)t_p/\lambda \tag{25.1}$$

and

$$\Delta_i \equiv (n_i - n_p)t_i/\lambda. \tag{25.2}$$

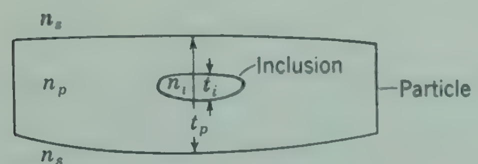

FIG. 20. Particle having an inclusion.

t_i is the thickness of the inclusion. t_p is the thickness of the particle as illustrated in Fig. 20. Δ is expressed as number of wave lengths.

o. *Refinements*

The above described methods for utilizing Dyson's interferometer microscope are open to so many extensions and refinements that it is beyond the scope of this chapter to dwell upon these important improvements in technique.

We shall restrict our discussion to an unpublished refinement that permits precision determination of Δ with the use of monochromatic illumination and that involves but a slight change in the interferometer. This change is illustrated in Fig. 21. A dielectric film having refractive index similar to that of plate *L* is deposited, as shown, to produce a sharp discontinuity of the amount *u* in δ between the portions *A* and *B* of the fully silvered spot, *F*. The background appears uniformly illuminated, i.e., regions *A* and *B* are equally bright, when the phase screw is set at the position $\delta = -u/2$, for then

$$\cos^2 \frac{\delta}{2} = \cos^2 \frac{\delta + u}{2}. \tag{26}$$

This monochromatic setting occurs near the white light position of the

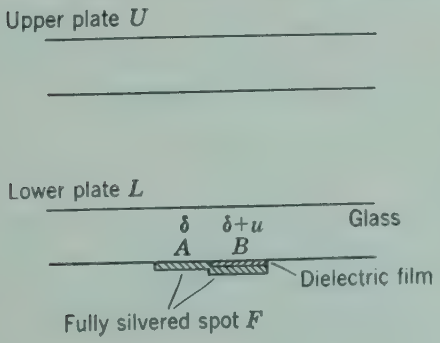

FIG. 21. A modification of the fully silvered spot in Dyson's interferometer microscope. A discontinuous phase step, *u*, amounting to a small fraction of one wave length is introduced between regions *A* and *B* of the fully silvered spot.

phase screw when u is a suitably small fraction of one wave length. The solutions δ of Eq. 26 are, in fact,

$$\delta_m = m - u/2; \qquad m = 0, 1, 2, 3, \text{etc.}; \tag{26.1}$$

$$\delta_\nu = \nu/2 - u/2; \qquad \nu = 1, 3, 5, \text{etc.}; \tag{26.2}$$

wherein δ and u are expressed as number of wave lengths of the monochromatic light. Eq. 26.1 and 26.2 correspond to bright and dark matches of the two regions A and B, respectively, of the background. These matches of illumination can be made critically because the illuminations of regions A and B vary oppositely as δ passes through the values of Eqs. 26.1 and 26.2. The dividing line between A and B disappears provided that the phase step between A and B is practically discontinuous.

These critical bright and dark matches allow precision calibration of the phase screw. The phase screw must be rotated so as to alter δ by one wave length between successive bright matches or successive dark matches. Thus, $\delta_{m+1} - \delta_m = 1$ and $\delta_{\nu+2} - \delta_\nu = 1$.

Let us consider the precision measurement of a small optical path difference Δ for a plate-like particle. We rotate the phase screw from the white light position in the direction, say, of increasing δ until regions A and B of the background appear equally dark and the dividing line disappears. At this phase setting, $\delta = \delta_1$, where

$$\delta_1 = 1/2 - u/2. \tag{26.3}$$

With the aid of the mechanical stage, we place the particle astraddle the dividing line as in Fig. 22. Then we rotate the phase screw from the position δ_1 to the nearest δ-value for which the dividing line disappears between regions C and D, Fig. 22. Let this δ-value be recorded as δ_a. One can show

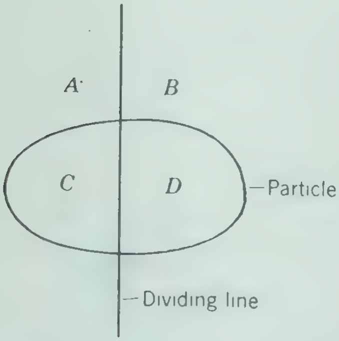

FIG. 22. Location of plate-like particles astraddle the dividing line between regions A and B of the stepped, fully silvered spot.

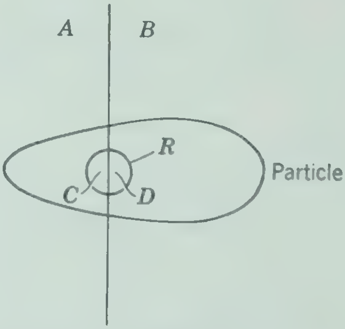

FIG. 23. Location of a region R, belonging to a nonuniform particle, astraddle the dividing line between regions A and B of the stepped, fully silvered spot.

from Eq. 20 that

$$\delta_a = \Delta + 1/2 - u/2$$

whence

$$\Delta = \delta_a - \delta_1 . \tag{27}$$

Further consideration shows that for other matches of regions C and D of the particle in the vicinity of the δ-value for any bright or dark match of regions A and B of the background

$$\Delta = \delta_a - \delta_{m \text{ or } \nu} . \tag{27.1}$$

As δ passes through any one of the discrete values δ_a for the match of regions C and D, the illuminations of these regions vary oppositely, as in the matching of regions A and B of the background. Therefore, both settings δ_a and $\delta_{m \text{ or } \nu}$ can be made critically. Under the most favorable conditions, it should be possible in this way to measure Δ within an error of ±0.01 wave length.

This method is not restricted to the measurement of plate-like particles. If Δ varies throughout the particle, the region R for which Δ is sought is placed astraddle the dividing line as in Fig. 23. One fixes his attention upon the local areas C and D within R and rotates the phase screw to the value δ_a for which the dividing line disappears between these two areas.

The method determines Δ together with its sign.

If Δ has been determined in any suitable way to the nearest integral number of half wave lengths, this method enables one to determine with precision the departure of Δ from the integral number of half wave lengths.

2. Multiple Beam Interference Microscopy

a. Introduction

Multiple beam interferometry is realized by splitting the incident monochromatic beam at each interreflection between two highly reflecting surfaces as illustrated in Fig. 24a. Surfaces A and B are assumed parallel. The emergent[34] rays 0, 1, 2, etc. reinforce one another to produce maximum brightness when the phase difference between these rays is an integral number of wave lengths. Correspondingly, at normal incidence the optical path AB plus the average of the two phase changes on reflection is an integral number of half wave lengths. Altering the optical path AB one half wave

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[34] A similar phenomenon occurs in the reflected beam. However, it will be seen that ray a is far stronger than rays b, c, d, etc. Consequently, contrast becomes poor unless ray a can be blocked. One cannot expect that ray a will be in phase with members b, c, d, etc. when the optical path AB plus the average of the two internal phase changes on reflection is exactly an integral number of half wave lengths.

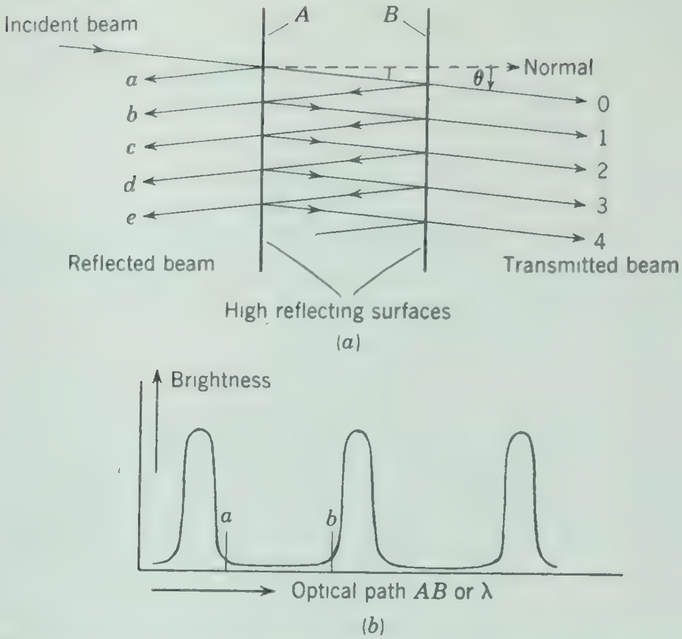

Fig. 24. The origin of the transmitted and reflected beams of the multiple beam interferometer is illustrated in Fig. 24a. The variation of the brightness or intensity of the transmitted beam as a function of the optical path AB or of λ is illustrated in Fig. 24b.

length leaves the brightness unchanged. The emergent rays interfere destructively to produce minimum brightness when the optical path AB plus the average of the two phase changes on reflection is an odd number of quarter wave lengths.

If the brightness of the emergent beam is plotted against the optical path AB or against wave length, the maxima are sharp and are separated by long dark spaces as in Fig. 24b. These maxima increase in sharpness as the reflectances of surfaces A and B approach unity. Silver, deposited by evaporation in vacuum, is the favored metal because it is capable of high reflectance together with notably low absorption.

Whereas the brightness is minimum midway between the maxima, a broad interval between points a and b, Fig. 24b, will appear dark. This interval broadens as the reflectance of surfaces A and B is increased. Accordingly, the optical path difference can vary considerably over a dark area in the image of a nonuniform particle. All bright settings are more critical than the dark settings and afford the higher precision in adjusting the separation of the reflecting plates, in measuring optical path differences, etc.

We may expect that particles having *small* optical path differences Δ will not appear in brightest contrast when the separation AB is adjusted for darkest background but, rather, when separation AB is adjusted at point b or a (Fig. 24b) according as $\Delta > 0$ or as $\Delta < 0$.

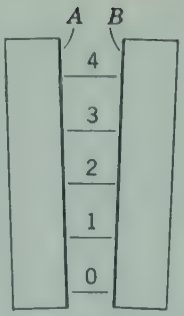

FIG. 25. Inclination of the reflecting interferometer surfaces A and B.

If the reflecting surfaces are inclined as in Fig. 25, bright fringes appear at points 1, 2, , etc. for which the optical path AB plus the average of the two phase changes on reflection is an integral number of half wave lengths. With small separations AB, the fringes appear to be localized in the wedge between surfaces A and B. The fringes describe loci of points having equal optical paths AB. Hence the fringes move in the direction 2-1 or 1-2 according as the separation AB is increased or decreased or according as the refractive index of the gap AB is increased or decreased. The bright fringes become more narrow as the reflectance of surfaces A and B is increased.

b. *Application to Topography*

The multiple beam interference microscope has long been in use by Tolansky[35] (1943; 1945) for measuring the heights of plateaus exhibited by surfaces such as mica. The surface to be examined and that of an optical flat are silvered by evaporation in vacuum to attain a reflectance in the range 90–96 per cent. The optical flat is laid upon the specimen (or conversely) to minimize the separation of the reflecting surfaces. For measuring the plateaus of crystallographic surfaces, the two reflecting surfaces are inclined slightly to produce straight, bright fringes. These fringes suffer a slight displacement S at the edge of a plateau. If W is the fringe width, i.e., the distance from one bright fringe to the next, the height of the plateau is given by $S\lambda/2W$ when the reflecting surfaces are separated by air. The fringes should be perpendicular to the edge of the plateau. No ambiguity in the interpretation appears so long as the height of the elevation is less than one quarter wave length. The interferometer consisting of the two silvered surfaces and the included gap is illuminated at normal incidence by a collimated beam of monochromatic light as illustrated in Fig. 26a. The microscope objective is focused upon the surface of the specimen. The details of the surface and the straight fringes appear in focus in the plane of the eyepiece.

When utmost precision is demanded in the measurement of the fringe displacement S/W, three primary precautions must be observed. The collimated beam must be restricted to rays that are practically parallel to the optical axis. This necessitates a small pinhole at the first focal plane of a

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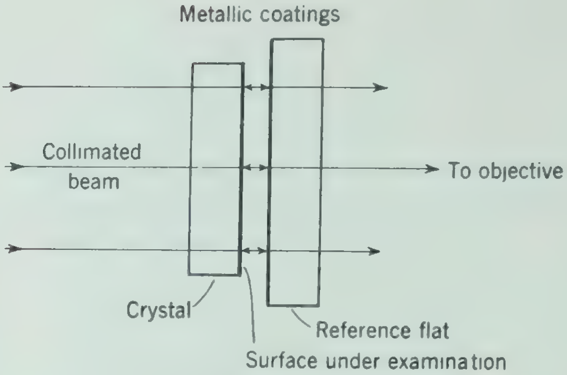
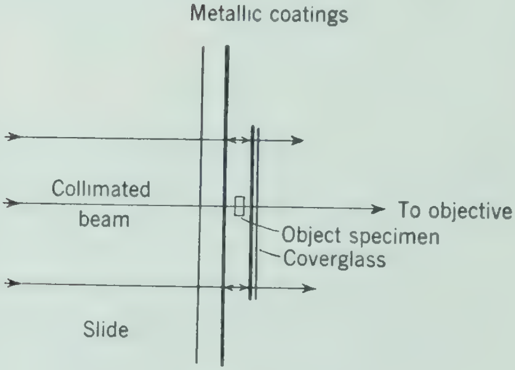
[35] It is not claimed that Tolansky made first use of the method.


Metallic coatings
Collimated beam
To objective
Crystal
Reference flat
Surface under examination

(a) Application to topography


Metallic coatings
Collimated beam
To objective
Object specimen
Coverglass
Slide

(b) Application to biology

Fig. 26. Location of the metallized surfaces in the applications to topography and biology. Figures 24a and 24b refer to the applications to topography and biology, respectively.

corrected substage condenser acting as collimator. The level of illumination becomes low. The separation of the two silvered surfaces must be small. This involves the danger of abusing the silvered coatings by pressing and sliding the reference flat against the specimen. Most important, the silvered coatings must have reflectance approaching unity as nearly as is possible practically. Objectives having high numerical aperture are advantageous because they enable one to measure safely the fringe displacement nearer to the point of displacement and because they transmit a greater portion of the diffracted and interreflected light emerging from the interferometer. The local surface irregularities of well polished optical flats have heights in the range 10 to 40 Å. The utmost precision is, accordingly, delimited to the vicinity of 10 Å unless unusual precautions are taken.

c. Application to the Biological Microscope

The adaptation of this method to the biological microscope is illustrated in Figs. 26B and 27. The upper surface of the slide and the lower surface of the cover plate are metalized by nontoxic metals such as inconel or titanium. The transmittance of each metalized surface is about 10 per cent. This adaptation lacks precision as compared to that attained in the observation of crystallographic surfaces. The inconel and titanium coatings have poor reflectance[36] as compared with silver. The surfaces of the slide and cover glass are not good optical flats. Moreover, the pinhole at the first focal plane of the collimator employed as substage condenser must be enlarged in order to obtain enough illumination. These compromises lead to broadening of the fringes that appear within the image of the particle. Fortunately, highest precision in the measurement of the optical path difference Δ is not needed. Consequently, the multibeam interference microscope has been used successfully, for example, by Mellors *et al.* (1953). Mellors has found that inconel and titanium are not unduly toxic and that standard slides and cover glasses have areas of sufficient flatness.

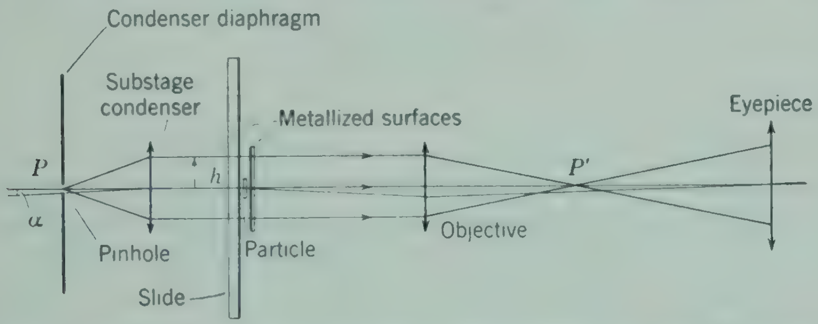


FIG. 27. Adaptation of multiple beam interferometry to the biological microscope.

The optical requirements of the substage condenser acting as collimator, Fig. 27, are not high. Thus a well constructed Abbe condenser should not exhibit excessive spherical aberration for rays emerging at heights h corresponding to microscopic fields of view. For the most exacting observations it may be advantageous to employ as collimator a microscope objective having a focal length of about 25 mm and a numerical aperture exceeding 0.1. If the numerical aperture of the collimator is too low, the entire field of

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[36] It is true that the reflectance of inconel or titanium can be increased by the deposition of quarter wave layers of dielectric films of alternatively low and high refractive index and that, perhaps, silver can be insulated from the specimen by means of a suitably thick layer of a nontoxic dielectric material. However, such additional coatings add excessively to the cost of the slide and cover plate.

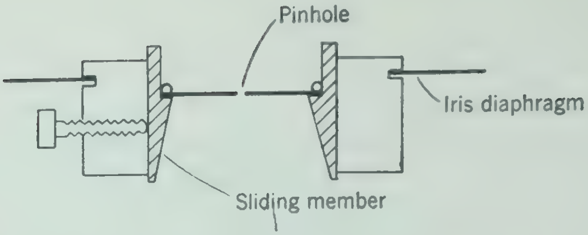

FIG. 28. An easily constructed adaptor that allows axial displacement of the pinhole.

view will not be illuminated. The front of the objective points toward the slide.

An easily constructed adaptor for mounting the condenser diaphragm in a conventional substage unit is illustrated in Fig. 28. This adaptor is supported by the leaves of the iris diaphragm and permits the pinhole to be slid into the first focal plane of the collimator.

d. *Adjustment*

After the light beam and substage unit have been centered upon the optical axis, the eyepiece is replaced by a telescope that has been focused upon a distant object. The centering telescopes supplied for adjusting phase microscopes are convenient since they may be focused for infinity. The objective is removed. As the condenser diaphragm is slid into the first focal plane of the substage collimator, the pinhole appears sharply focused in the telescope. A single pinhole in this adjustment serves for the whole series of microscope objectives. The objective and the eyepiece are replaced.

e. *Allowable Radii of the Pinhole*

The penalties incurred by the choice of too large a pinhole are loss of contrast, excessive broadening of the fringes and, finally, invalidation of the usual interpretation of the observations. The maximum allowable radius of pinhole is decreased by increasing the separation of slide and cover glass, by decreasing the lateral dimensions of the particle and by increasing the variation of the optical path difference Δ between the particle and its surround.

Experimentally, one may begin by inserting a pinhole so small that the fringes are barely visible and by observing the location and number of fringes within a typical particle. The separation of the slide and cover plate should be typical also. The diameter of the pinhole may be increased so long as the location and number of the fringes remain practically unaltered. The final compromise usually involves selecting the smallest pinhole that provides minimal acceptable illumination. Diameters of 0.25, 0.5, 1 and 2 mm are useful with substage collimators having focal lengths of about 25 mm. The larger diameters 1 and 2 mm may not be found safe with the smallest particles or with the largest separations of slide and cover plate.

We shall discuss briefly the two main physical considerations that delimit the size of pinhole. We may neglect the effects of the phase changes that occur upon reflection at the metalized surfaces.

With respect to Fig. 29, the half-angle α subtended by the pinhole at the collimator is related by Snell's law to the angle θ between the surface normal and the rays within the gap AB such that

$$\sin \alpha = n_s \sin \theta \tag{28}$$

where n_s is the refractive index of the gap.

Let N denote the number of half waves in the separation d of the metalized surfaces when the rays are incident normally. The light flux emerging in the direction of the normal is maximum provided that

$$n_s d = N\lambda/2. \tag{29}$$

If θ is increased from $\theta = 0$ to the value for which

$$n_s d \cos \theta = (N - 1/2)\lambda/2, \tag{30}$$

the light flux emerging along the α-direction from the normal is zero. Certainly, angles θ as large as those given by Eq. 30 cannot be tolerated because rays having both maximum and minimum flow of energy emerge from the slide.

Suppose that instead of Eq. 30 we write

$$n_s d \cos \theta = (N - 1/p)\lambda/2. \tag{31}$$

Then $n_s d - n_s d \cos \theta = \lambda/2p$ so that as θ is increased from zero to the value given by Eq. 31, the phase difference between successive rays 1, 2, 3, etc., Fig. 29, is decreased by the amount $\lambda/2p$. Let Eq. 31 be divided by Eq. 29. The result is

$$\cos \theta = \frac{pN - 1}{pN}. \tag{32}$$

As our criterion, therefore, we must delimit $\theta_{\max}$ so that

$$\theta_{\max} \leqq \cos^{-1} \frac{pN - 1}{pN} \tag{33}$$

wherein N is the number of half waves between the metalized surfaces at normal incidence. The choice $p = 20$ should prove amply large for most purposes of interference microscopy.

As N approaches infinity, $\theta_{\max}$ approaches zero. This means that the pinhole diameter must be reduced toward zero as the separation of slide and cover glass becomes large.

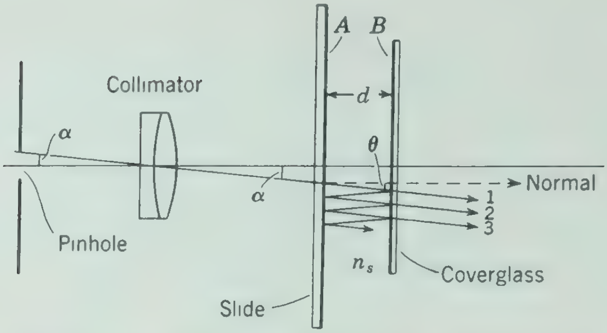

FIG. 29. The paths of oblique rays through the interferometer. The normal to the surfaces A and B is assumed to be parallel to the optical axis.

The number $N = 60$ is large but is not uncommon unless precautions are taken to obtain small separations between slide and cover glass. With $p = 20$ and $N = 60$, $\theta_{\max} = 0.041$ radians. If $n_s = 1.33$, $\alpha_{\max} = 0.054$ radians. If the focal length of the substage collimator is 25 mm, the maximum radius of the pinhole is $25 \times 0.054 = 1.4$ mm. Of course, p must be chosen larger than 20 to meet the precision required, for example, in the topographical measurements performed by Tolansky.

The second physical consideration requires that a sufficiently large number of interreflected rays shall pass through each sensibly uniform portion of the particle. We shall see that this requirement imposes the greater delimitation upon the size of pinhole.

In Fig. 30, θ is so large that only three interreflections pass through the particle. Moreover, the pairs of interreflected rays pass through regions having different Δ since the particle is not uniformly thick. Consequently, the emergent rays 2, 3, 4 are too few in number and do not have a common path difference. The physical situation depicted in Fig. 30 would be unfortunate.

Since θ must be small, the displacement D (see Fig. 30) is given with excellent approximation by

$$D = 2\,d\theta \tag{34}$$

where d is the separation of slide and cover glass. Let s denote the smallest number of interreflections that must pass through a sensibly uniform element of the particle. Then the length L of this element must obey the requirement

$$L > sD = 2s\,d\theta$$

or, conversely, θ must obey the criterion

$$\theta_{\max} \leqq L/2s\,d \tag{35}$$

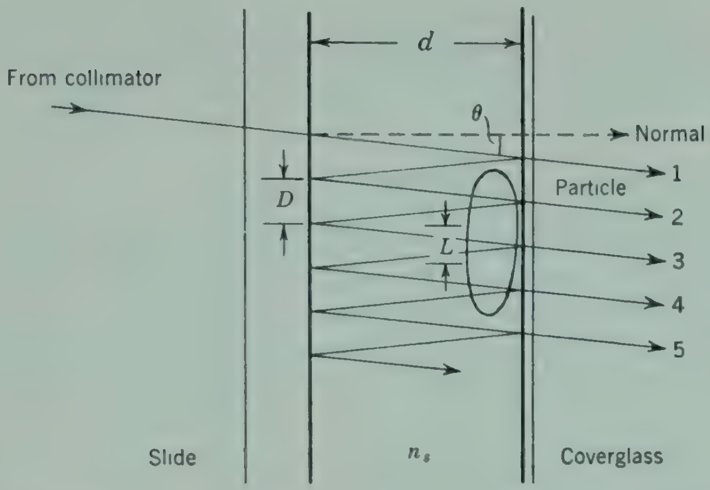

FIG. 30. Passage of interreflected, oblique rays through a nonuniformly thick particle.

The corresponding radius $r_{\max}$ of the pinhole is given with good approximation by the equation

$$r_{\max} = F_c n_s \theta_{\max} \leqq F_c n_s L/2s\,d \tag{36}$$

where F_c is the focal length of the collimator, n_s is the refractive index of the medium between slide and cover glass.

The choice of the smallest acceptable number s is difficult. With metalized coatings of inconel or titanium, whose reflectance is relatively low, the choice $s = 4$ or 5 may be realistic.

As an example, let $L = 1\mu$, $d = 20\mu$, $2s = 10$, $n_s = 1.33$ and $F_c = 25$ mm. Then $r_{\max} \leqq 0.17$ mm. If d is reduced to 10μ, the maximum radius of the pinhole can be increased to 0.34 mm. Unless L is quite large, Eq. 36 requires a smaller pinhole than does Eq. 33, i.e., the requirement that an adequate number of interreflections shall pass through sensibly uniform elements of the particle can ordinarily be expected to impose the greater delimitation upon the size of pinhole.

f. Measurement of Thin Plate-Like Particles

Optical path differences Δ between plate-like particles and surround are easily measured without ambiguity provided that $|\Delta|$ is less than one quarter wave length. The metalized surfaces A and B are preferably inclined at a small angle β, see Fig. 31a, to form straight fringes as illustrated in Fig. 31b. The width W of these bright fringes is measured together with the fringe shift S. Then

$$|\Delta| = S/2W \tag{37}$$

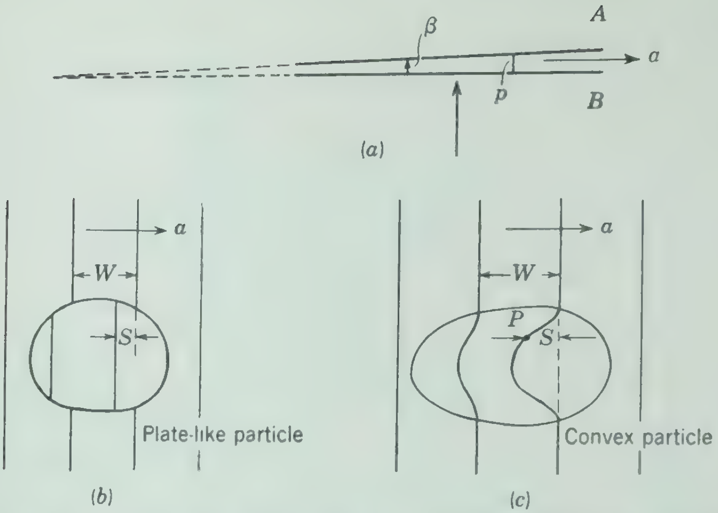

FIG. 31. The formation and movement of narrow interference fringes produced by interreflection between two inclined interferometer surfaces A and B.

with

$$\Delta \equiv (n_p - n_s)t/\lambda \tag{37.1}$$

n_p and n_s are the refractive indices of the particle and surround, respectively. t is the thickness of the particle.

The sign of Δ can be determined in the following way. With reference to Fig. 31a, the fringes move toward the open end of the wedge along the arrow direction a as plates A and B are pressed more closely together. Each fringe demarks the locus of equal optical paths p. Each fringe moves so as to maintain its associated optical path whether one of the surfaces is moved as in Fig. 31a or whether the fringe passes across a particle as in Fig. 31b. Accordingly, the fringe shift S will be along or against the arrow direction a according as $n_p < n_s$ or $n_p > n_s$. Fig. 31b illustrates the more common case $n_p > n_s$.

Ambiguities as to direction and magnitude of fringe shift arise when $|\Delta| \geqq \frac{1}{4}$ wave length. It is often possible to resolve these ambiguities by measuring the fringe shift for several well separated wave lengths because the fringe shift increases with decreasing wave length.

g. *Measurement of Geometrically Convex Particles*

When the variation in Δ exceeds one half wave length, it is preferable to adjust the metalized surfaces into parallelism over the area containing the particle. Since the separation of slide and cover glass is more critical for the bright backgrounds, this separation should be adjusted for *maximum* illumination of the background. The optical path difference Δ between the bright background and the location of the first, bright, ring-shaped fringe within the image of the particle is one half wave length. Consecutive locations of bright fringes are likewise separated in optical path by the amount $\lambda/2$. Hence

$$|\Delta|_{\max} = N/2 \text{ wave lengths} \tag{38}$$

where N is the number of bright fringes counted within the image of the particle. Eq. 38 is exact whenever the N^{th} bright fringe happens to occur as a bright spot centered about the thickest portion of the particle. This inner spot may be bright, dark or grey. The locations of adjacent bright and dark fringes are separated in optical path by the amount $\lambda/4$. Therefore,

$$|\Delta|_{\max} = N/2 + \tfrac{1}{4} \text{ number of wave lengths} \tag{38.1}$$

if the inner fringe is dark. If the inner fringe is neither brightest or darkest and if high precision is demanded, one may vary the wave length by means of a monochromator or a rotatable, narrow band-pass interference filter to find the wave length at which the inner fringe is brightest or darkest. With change of wave length, the separation of the plates must be readjusted to maintain maximum illumination of the background.

If m is the number of the bright fringe counted from the edge of the image of the particle, then at the location of this m^{th} fringe

$$|\Delta|_m \equiv |n_p - n_s|\, t_m/\lambda = m/2 \tag{39}$$

where t_m is the thickness of the particle at the location of the m^{th} fringe. If $n_p - n_s$ and λ are known, it follows that

$$t_m = m\lambda/2\,|n_p - n_s|\,. \tag{39.1}$$

In this way, Δ and the thickness t can be measured at many points distributed throughout the particle. The number of measured points can be increased by using more than one wave length.

The sign of Δ can be determined by decreasing[37] the separation of the slide and cover glass. If $n_p > n_s$, the fringes contract about the thickest portion of a geometrically convex particle whereas they expand about this thickest portion when $n_p < n_s$.

When $|n_p - n_s|$ is small, $|\Delta|_m$ and m become small also. The number of fringes within the image of the particle becomes low. The number of measured t-values may therefore become inadequate for determining the volume of the particle. Mellors *et al.* (1953, pp 375, 378 and 382) avoid this difficulty in the measurement of embryonic fibroblasts and sarcoma cells by surrounding these particles by air instead of aqueous solution. The optical path difference (and hence the number of fringes) thus obtained is 4 to 17 times greater than that with protoplasm mounted in aqueous solution.

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[37] This is usually accomplished by pressing upon the cover glass.

When the product $| n_p - n_s | t/\lambda < \frac{1}{2}$, the method of parallel metalized surfaces fails. Fortunately, in such cases the method of inclined metalized surfaces is applicable to convex particles. In fact, the method of inclined surfaces can be used successfully with geometrically *convex* particles even when $|n_p - n_s | t/\lambda > \frac{1}{2}$. This method has been discussed in subsection *f*. The application to a convex particle is illustrated in Fig. 31c. With geometrically convex particles, the fringe displacement $S = 0$ at the edge of the image of the particle and is maximum at some point on the curved fringes that appear within the particle. To determine[38] $| \Delta |$ at a point P, one measures S and W as described by Fig. 31c and utilizes Eqs. 37 and 37.1 to interpret the measurements for $| \Delta |$ and t. Of course, $n_p - n_s$ must be known independently in order to compute the thickness t at point P. Fringe displacements $S > W$ can be measured so long as one is able to trace the path of the curved fringe. With small particles it may become necessary to decrease the fringe width W when S is large. The fringe width is decreased by increasing the angle β, Fig. 31a, between the metalized surfaces A and B. Points P within the entire particle may be explored by altering the separation of the metalized surfaces together with the angle of inclination β.

h. Particles Having Thick Edges

The multiple beam interference microscope is impractical for measuring large optical path differences $| \Delta |$ unless it is possible to count fringes from the edge of the particle. At the abrupt edges of thick particles the number of fringes may be too great to be counted successfully. This fringe count can be avoided with Dyson's interference microscope by utilizing the properties of the white light fringe, a phenomenon that the multiple beam interference microscope does not possess.

It is possible to avoid the fringe count at the abrupt edges of thick particles by illuminating the multiple beam interference microscope with white light and by employing a method known as channel spectra. This method requires the use of a wave length spectrometer and increases the difficulties of interpreting the observations.

i. Measurement of Volume

Interference microscopy permits the determination of the volume of *optically homogeneous* particles when $n_p - n_s$ is known. We have seen how the thickness t can be determined at points distributed over the area of the particle. A family of smooth curves may be drawn through this set of t-values to ascertain the function $t(x, y)$ where x, y are the coordinates of the points in the plane of the slide. The volume V of the particle is now given by the double integral.

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[38] The sign of Δ can be determined from the direction of the fringe shift within the image of the particle in the manner already described in sub-section *f*.

$$V = \iint t(x, y)\, dx\, dy = \frac{\lambda}{n_p - n_s} \iint \Delta(x, y)\, dx\, dy \tag{40}$$

or by the single integral

$$V = \int t(A')\, dA = \frac{\lambda}{n_p - n_s} \int \Delta(A')\, dA \tag{41}$$

in which the integration extends over the area of the particle. $t(A')$ denotes, for example, the value of t at the point x, y about which the element of area dA is centered.

The task of solving these integrals can be reduced greatly if, for example, the particle has axial symmetry about an axis parallel to the optical axis of the microscope. In this case

$$V = \frac{2\pi\lambda}{n_p - n_s} \int_0^R \Delta(r) r\, dr \tag{42}$$

where R is the radius of the particle in the plane of the slide. The line element dr lies in the plane of the slide. Hence it suffices to determine Δ at several radial points r, to plot these Δ-values against r for determining a smooth curve $\Delta(r)$, to plot the product $r\Delta(r)$ against r and finally to determine with the aid of a planimeter the area under the curve $r\Delta(r)$ between the points $r = 0$ and $r = R$.

Convenient approximations to the integral of Eq. 41 have been described by Mellors *et al.* (1953, pp 373–374).

Since the density of protoplasm is nearly unity, the hydrous mass in grams of a particle composed of protoplasm may be set equal to the volume of the particle expressed in *ml*.

j. The Measurement of Anhydrous Mass

Protoplasm may be regarded as a protein solution having refractive index n_p. If the refractive index of the solvent is n_s,

$$n_p - n_s = \alpha c \tag{43}$$

where α is a constant called the specific refractive increment and c is the concentration in number of grams of anhydrous protein per 100 ml of solution. We consider the particle *homogeneous* with refractive index n_p given by Eq. 43 and consider the surround *homogeneous* with the refractive index n_s of the solvent. Then the optical path difference Δ between particle and surround is given as number of wave lengths by

$$\Delta = (n_p - n_s)t/\lambda = \alpha ct/\lambda \tag{44}$$

where t is the thickness of the particle.

The mass M_a of anhydrous protein within the particle is given by

$$M_a = \frac{c}{100} V = \frac{n_p - n_s}{100\alpha} V \tag{45}$$

where V is the volume of the particle. It follows from Eqs. 41 and 45 that

$$M_a = \frac{\lambda}{100\alpha} \int_{\text{area of particle}} \Delta(A')\, dA \tag{46}$$

If the particle is plate-like so that $\Delta(A') = \text{constant} = \Delta$,

$$M_a = \frac{\lambda\Delta}{100\alpha} A \tag{46.1}$$

where A is the area of the particle in the plane of the slide. If the particle possesses axial symmetry as in the assumptions leading to Eq. 42,

$$M_a = \frac{2\pi\lambda}{100\alpha} \int_0^R \Delta(r) r\, dr \tag{46.2}$$

where R is the radius of the particle in the plane of the slide and the radial line r is measured from the center of the particle in the plane of the slide. The data for determining the smoothed curve $\Delta(r)$ may be obtained by means of Dyson's interference microscope or by means of the multiple beam interference microscope. The results presented as Eqs. 46 have been applied in specialized form by Mellors *et al.* (1953, pp 374–375).

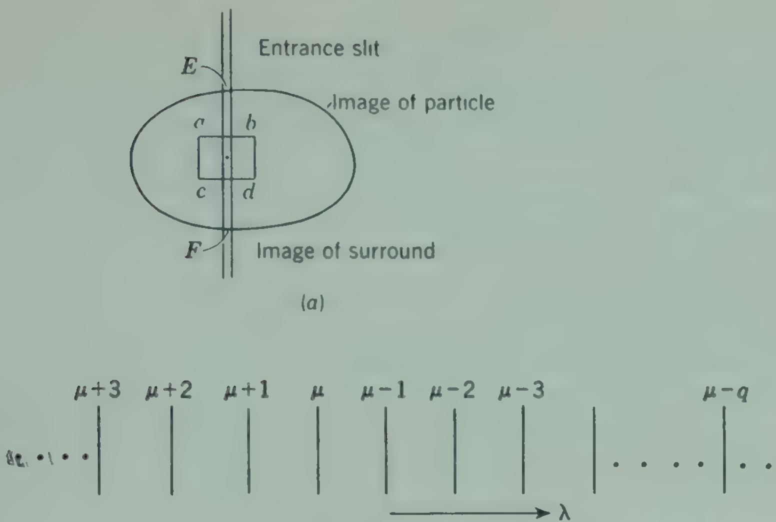
k. The Method of Channel Spectra

The method of channel spectra is employed to greatest advantage with a multiple beam interference microscope operated under the conditions of narrow-coned axial illumination as illustrated in Fig. 27. The metallized surfaces are adjusted for parallelism.

The particle is focused sharply by the microscope upon the entrance slit of a calibrated spectrometer, i.e., of a wave length spectrometer. The entrance slit is imaged in the second focal plane of the spectrometer telescope. This image is viewed through an eyepiece.

Ordinarily, the image of the particle will straddle the entrance slit as illustrated in Fig. 32a. The microscope is illuminated by white light.

Let us consider first the appearance of that portion of the entrance slit which intercepts the image of the surround. The multiple beam interferometer transmits, as we have seen, a narrow spectral band centered



(a)

(b) μth spectral band

Fig. 32. The method of forming channel spectra is illustrated in Fig. 32a. The appearance of the straight channel spectra that correspond to the image of the surround is illustrated in Fig. 32b.

about every wave length λ_ν obeying the relation

$$2n_s d + \phi_\nu = \nu\lambda_\nu \tag{47}$$

where n_s is the refractive index of the surround, d is the separation of the slide and cover glass, ν is an integer and ϕ_ν is the sum of the phase changes upon reflection at the two metalized surfaces at the wave length λ_ν. The prism disperses these spectral bands in the focal plane of the eyepiece. The bands become narrower as the reflectance of the metalized surfaces is increased and as the width of the entrance slit is decreased. As one rotates the microscope slide about the optical axis, the spectral bands remain parallel to the geometrical image of the entrance slit only when the metalized surfaces of the slide and cover glass have been adjusted into parallelism. The appearance of the spectral bands is illustrated in Fig. 32b.

The order number ν of any selected spectral band can be found as follows. One measures λ_ν together with $\lambda_{\nu-q}$ of the q^{th} neighboring band on the long wave length side of λ_ν. Then from Eq. 47

$$2n_s d + \phi_\nu = \nu\lambda_\nu\,;$$

$$2n_s d + \phi_{\nu-q} = (\nu - q)\lambda_{\nu-q}$$

If $\lambda_{\nu-q}$ is not far different from λ_ν, we can expect that $\phi_{\nu-q} - \phi_\nu \rightarrow 0$. Whence, approximately, $(\nu - q)\lambda_{\nu-q} = \nu\lambda_\nu$ and

$$\nu = \frac{q\lambda_{\nu-q}}{\lambda_{\nu-q} - \lambda_\nu}. \tag{47.1}$$

If the dispersion of the phase change ϕ on reflection is in fact negligible, the computed right-hand member of Eq. 47.1 will fall near a particular integral value irrespective of the choice of q. (q is counted negative on the short wave length side of λ_ν.) Under these circumstances the order number ν is this particular integral value. In measuring λ_ν and $\lambda_{\nu-q}$, it is essential that the optical path $n_s d$ remains constant. Drifts that change ν by ±1 or even ±2 are likely to occur when one does not allow the optical path to assume equilibrium after inserting the slide into the microcope.

The problem of determining ν within an error of unity is difficult when the dispersion of ϕ is neither negligible nor known independently.

Let us consider next the appearance of that portion of the entrance slit which intercepts a small area about a point P in the image of the particle. This area can be examined, for example, by blocking or by ignoring the sections of the entrance slit above *ab* and below *cd* in Fig. 32a. If the height *bd* is small enough and if the slit width is narrow enough relative to the magnified image of the particle, the dispersed spectral bands corresponding to the small area *abcd* will be centered about wave lengths λ_μ such that

$$2n_s d + 2\Delta + \phi_\mu = \mu\lambda_\mu \tag{48}$$

where μ is an integer. Δ is the optical path difference between particle and surround measured about point P within the projected area of the particle. In dealing with channel spectra, we shall prefer to express Δ as microns, i.e., as having the dimension of optical path rather than as the dimensionless quantity of phase. The central wave lengths λ_μ will be more difficult to measure than the central wave lengths λ_ν in the straight bands belonging to the background.

The order number μ can be determined by a procedure similar to that for determining the order number ν of the surround with the aid of Eq. 47.1. Thus

$$\mu = \frac{p\lambda_{\mu-p}}{\lambda_{\mu-p} - \lambda_\mu} \tag{48.1}$$

where $\lambda_{\mu-p}$ is the measured wave length of the p^{th} spectral band on the long wave length side of the measured wave length λ_μ. It is assumed that the dispersions of ϕ and of the refractive indices of the particle and surround are negligible. If these dispersions are negligible, the μ's computed from Eq. 48.1 will fall near a particular integer irrespective of the choice of p. The departures from this integral order number μ will then be due to errors in the measurement of wave length. It is preferable to select λ_μ near λ_ν in order that $\phi_\nu - \phi_\mu$ shall be small.

By subtracting Eq. 47 from Eq. 48 and setting $\phi_\mu - \phi_\nu = 0$, we obtain the approximate solution

$$\Delta = \tfrac{1}{2}(\mu\lambda_\mu - \nu\lambda_\nu) \tag{49}$$

where Δ appears in microns when λ is expressed in microns. If Δ is desired in the dimensionless quantity of phase, expressed as number of wave lengths for the wave length λ_0, one writes

$$\Delta = \frac{1}{2\lambda_0}(\mu\lambda_\mu - \nu\lambda_\nu). \tag{49.1}$$

The above procedure can be repeated to determine Δ at several points P. However, the *variation* of Δ throughout the particle can be obtained more simply and with less labor by direct observation with monochromatic illumination in the multiple beam interference microscope.

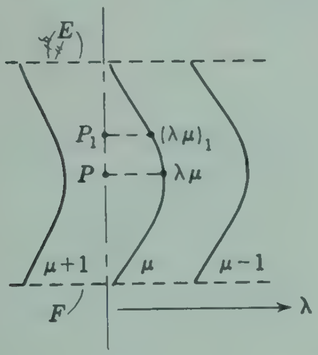


FIG. 33. The curved channel spectra corresponding to the projected image of particles having variable optical path difference with respect to their surround.

Channel spectra are convenient[39] for measuring Δ along a line through the particle, i.e., along the line EF of Fig. 32a. If the particle varies in thickness along the line element EF intercepted by the slit, the spectral bands corresponding to the intercepted portion EF bend as illustrated in Fig. 33. Each curved band has a fixed order number, μ, $\mu \pm 1$, etc. and obeys Eq. 48. We conclude from Eq. 48 that the spectral bands bend toward increasing or decreasing wave lengths according as Δ is increased or decreased. The values Δ_1 at points P_1 are related to Δ at point P such that

$$2n_s d + 2\Delta + \phi_\mu = \mu\lambda_\mu\,;$$

$$2n_s d + 2\Delta_1 + (\phi_\mu)_1 = \mu(\lambda_\mu)_1\,.$$

If the dispersions of n_s and ϕ are negligible, it follows by subtraction that

$$\Delta - \Delta_1 = \frac{\mu}{2}[\lambda_\mu - (\lambda_\mu)_1]. \tag{50}$$

Since μ must be determined before computing Δ from Eq. 49.1, the additional values Δ_1 along the line EF are obtained at relatively little additional labor.

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[39] Provided that the variation in Δ does not exceed one half wave length.

The simplest interpretation leading to Eq. 50 does not hold for all points P_1 along the line EF when the variation in Δ exceeds $\lambda_\mu/2$; for then the order number μ assumes more than one integral value along the line EF. Consequently new spectral bands, displaced along EF, appear. When the order number passes through several integral values across the particle, the variation in optical path difference is more simply determined by observing the fringes formed within the image of the particle in the multiple beam interference microscope.

It is beyond the scope of this chapter to discuss the many potentially useful variations in the technique of applying channel spectra to the measurement of biological particles. The method is laborious but possesses considerable flexibility.

3. POLARIZATION METHODS

Birefringent elements serve as beam-splitters because they divide the incident beam into two beams[40] polarized at right angles. A large number of adaptations to the interference microscope are possible.

Dyson (1953) has described a modification of Lebedeff's microscope (1930) wherein the interferometer plates L and U of Fig. 13 are replaced by unsilvered birefringent plates. The optical axes of these two plates are located in the plane of the plate and are oriented at right angles. The beam incident upon the lower plate L is, presumably, plane polarized at 45° with respect to the optical axis. One polarized image of the source of light is focused upon the object, but the second polarized image of the source is displaced axially from the plane of the object. The upper birefringent plate serves to reunite this pair of orthogonally polarized images at the plane of the eyepiece where the two beams are caused to interfere by passage through an appropriately located analyzer. The phase difference between the two interfering beams (and hence the optical path difference Δ) is determined in a sensitive manner with the aid of additional birefringent elements that act in conjunction with the analyzer.

Philpot (1951) described a related adaptation to the interference microscope in which the birefringent beam-splitting and restoring elements are incorporated as lens components in the substage condenser and microscope objective. Chas. Baker Ltd. exhibited[41] an interference microscope similar to Philpot's microscope but having the two birefringent elements incorporated as plates in the substage condenser and microscope objective.

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[40] Corresponding to the ordinary and extraordinary rays.

[41] Symposium and exhibition organized by the Royal Microscopical Society in collaboration with other groups and opened at King's College, London, March 20, 1953.

In still other variations of the polarization method, the two birefringent elements consist of Rochon prisms that serve first to separate and then to reunite the orthogonally polarized beams angularly.

These polarization methods contain analyzing elements that enable one to control the degree of contrast in the image and to measure the optical path difference between the particle and surround. As with Dyson's interferometer microscope of Fig. 13, the phase of the reference beam[42] will be modified by passage of this beam through an excessive number of particles located beyond the field of primary interest.

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[42] In this case, the beam that is not focused or centered upon the object.

Chapter 8

BIREFRINGENCE AND DICHROISM

Gerald Oster

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I. Nature of Polarized Light

Light may be regarded as a propagated electrical disturbance in which an electric field is oscillating in a direction perpendicular to the direction of propagation of the light. In ordinary unpolarized light the direction of the vibration may be in any direction in the plane normal to the direction of propagation. With plane polarized light, however, the direction is specified. This direction together with the direction of propagation constitutes a plane, the plane of vibration.

In this chapter we shall be concerned mainly with the passage of plane polarized light through optically anisotropic media, i.e., through substances which exhibit two refractive indices (more than two in the case of certain crystals) or two coefficients of light absorption. The former materials we refer to as being double refracting or birefringent. Birefringent systems are extremely common in biology and a quantitative investigation of birefringent material by means of polarized light gives us a better understanding of the ultrastructure of such materials. The majority of crystalline substances are optically anisotropic and an examination by means of polarized light in the polarizing microscope can lead to a rapid identification of the crystals. Dichroic materials, that is, substances which have two coefficients of absorption in different directions, are more commonly encountered in biological materials than is generally supposed. Although the number of substances which are dichroic in the visible range, e.g., crystalline hemoglobin, are limited, nearly all oriented fibrous materials will exhibit a dichroism in the ultraviolet or infrared regions of the spectrum. The techniques of observing dichroism have been known for several years but systematic investigation of dichroism in biological structures have only been carried out very recently and offers a powerful method for the illucidation of the molecular structure.

When polarized light, produced by one of the polarizers described in the section below, is made to pass through another polarizer (called the analyzer) the light is extinguished if the plane of vibration of the analyzer is at right angles to that of the first polarizer. That is, we obtain no light through two crossed polarizers. If, on the other hand, the polarizers are at angles other than 90° we obtain some transmission of light, the intensity of light being a maximum at 0°, i.e., the polarizers are parallel. The intensity of transmitted light I is given by the law of Malus, namely $I = I_0 \cos^2 \theta$ where θ is the angle between the planes of polarization of the polarizer and the analyzer and I_0 is the intensity of the transmitted light when they are parallel ($\theta = 0°$). Note that the intensity is reduced to one-half (half shade) when $\theta = 45°$. The law of Malus can be written in alternative form, namely $\Delta I/I = 2 \tan \theta \Delta\theta$ where ΔI is the change in intensity with change in angle $\Delta\theta$. The human eye can accurately determine values of $\Delta I/I$ if I is sufficiently low (the Weber-Fechner law) and hence the eye is particularly sensitive to slight changes in θ when the polarizers are nearly crossed since $\tan \theta$ approaches infinity as θ approaches 90°. It is this principle which governs the design of all visual polarimeters.

So-called optically active substances have the power of rotating the plane of polarization of polarized light. For a given optically active substance, the degree of rotation will be proportional to the thickness of the sample. The degree of rotation will also depend on the wave length of the light (rotatory dispersion). For example, for crystalline quartz, the angle of rotation for blue light is nearly three times that for green light. The study of optical activity is very important in biochemistry particularly in the examination of solutions of carbohydrates and amino acids. This subject lies out of the scope of the present chapter and we refer the reader to comprehensive treatments of optical activity (for example, Lowry, 1935; Heller, 1949; Partington, 1953).

When a beam of light is incident upon a birefringent crystal it is generally split into two plane polarized beams with vibration planes at right angles. One beam is called the ordinary ray and obeys the ordinary laws of refraction and the other beam is designated as the extraordinary ray (Fig. 1). This is the basis for the construction of the Nicol prism described below. The two beams travel at unequal rates in any specified direction. Because of the unequal velocities the beams will differ in phase and therefore should be expected to interfere when they are made to recombine into the same plane of vibration. The value of this difference in phase, for any given kind of light, depends on the difference of the velocities and on the length of the path through the crystal. The velocity of light is inversely proportional to the refractive index so that the optical path difference is $\Delta = (n_e - n_0)t$ where n_e and n_0 are the refractive indices for the extraordinary and ordinary rays, respectively, and t is the thickness of the crystal. The phase difference is given by $\delta = \frac{2\pi}{\lambda}\Delta$ where λ is the wave length of the incident light. Destructive interference will occur when the waves are 180° (half wave length) out of phase or an odd integral multiple thereof, i.e., when $\delta = m\pi$, $m = 1, 3, 5, \ldots$. We shall show later how interference of polarized light can be used to measure extremely small differences in refractive indices.

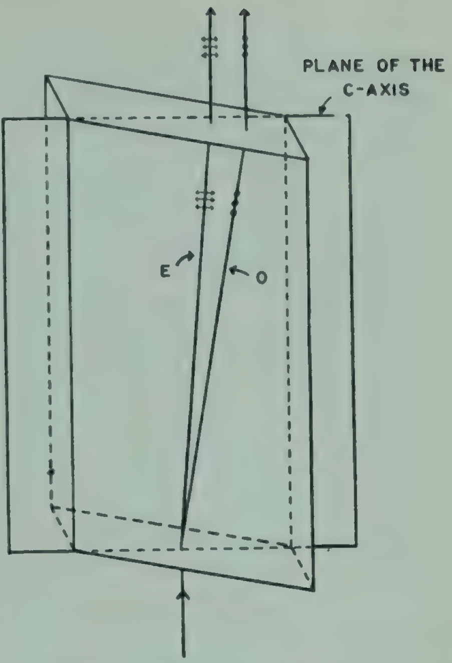

FIG. 1. Passage of light through calcite.

II. Production of Plane Polarized Light

Unpolarized light may be rendered plane polarized in four ways: 1) by the scattering of light from a system of small particles, 2) by reflection at a polished surface at an appropriate angle, 3) by double refraction of light by a Nicol prism or some modification of this prism, and 4) by selective absorption by a dichroic material such as Polaroid.

1. SCATTERING

When unpolarized light is made to pass through a system containing particles which are small compared with the wave length of the incident light, the system will exhibit Rayleigh scattering (see Chapter 2). If the particles are optically isotropic, the scattered light when viewed at right angles will be completely plane polarized in the vertical direction. If a given system does not exhibit completely plane polarized scattered light when observed at right angles to the incident beam, it may be deduced that the particles are either not optically isotropic or are not small or both. Lack of complete polarization means that there is a horizontal component of the polarized light and the ratio of these two components, the depolarization, gives information regarding the structure of simple molecules (see, for example, Oster, 1948, Sections II A2 and II B4).

It is usually not practical to produce polarized light by scattering on a laboratory scale since the intensity is very low. Light from the sky is two-thirds vertically polarized at an angle of 90° removed from the direction of the sun. The plane of vibration of a polarizer can be determined by noting the position where the light from the sky shows maximum extinction. In this orientation the direction of vibration of the polarizer is given by the horizontal direction. It has been proposed (von Frisch, 1950) that bees are guided in flight by the determination of the depolarization of the scattered light from the sky since the eye of the insect contains dichroic structures (see below).

2. Reflection

Polarization by reflection is achieved by reflecting a beam of ordinary light off a polished sample of a dielectric (a nonconductor) such as glass. If the incident beam falls at an angle of incidence (with respect to the axis normal to the sample), Brewster's angle i, given by $\tan i = n$ where n is the refractive index of the sample, then the reflected light will be polarized with its plane of vibration parallel to the surface of the reflector. For glass n equals about 1.54 so that the incident light falling on a glass slide should be at about 57° to the normal. The polarization of the transmitted light is more nearly complete if a pile of microscope slides are used so that the reflections are enhanced. The plane of vibration of a polarizer can be determined by observing the reflection from a glass slide and rotating the polarizer until extinction is achieved. The plane of vibration of the polarizer is now perpendicular to the surface of the reflector. Metallic reflecting surfaces are not suitable for this test since they do not produce plane polarized light in the visible region.

Polarized visible light is generally not produced by reflection nowadays. However, polarized infrared is usually produced in this manner. For this purpose a pile of polished selenium or silver chloride plates are used (Pfund, 1904; Elliott, Ambrose, and Temple, 1948).

3. Double Refraction

The first experiments on polarized light nearly three hundred years ago were carried out with calcite (calcium carbonate), which occurs naturally in the form of large and very transparent crystals (Iceland Spa). Light passing through calcite is split into two beams as shown in Fig. 1. The ordinary ray obeys the simple laws of refraction and reflection, that is, toward it the crystal behaves like an isotropic solid. Only when the extraordinary ray passes through a certain direction in the crystal, the optic axis, it may be said to have a refractive index. For calcite the difference in refractive index of the two rays, the birefringence, is very great ($n_e - n_0 = -0.1721$). It is this property which makes calcite especially useful. The birefringence of sodium nitrate is even greater ($n_e - n_0 = -0.2513$) but these crystals do not occur naturally. If the calcite crystal is rotated about the incident beam the ordinary ray remains stationary but the extraordinary ray rotates (Fig. 1). For calcite the distance of separation of the two beams is approximately one-tenth the thickness of the crystal.

The calcite crystal may be cut in a variety of ways and recombined to give prisms used for the production of polarized light, the most important of which is the Nicol's prism ("Nicol"). This prism which is described in detail in all textbooks on optics produces one ray, the extraordinary ray, of plane polarized light, the ordinary ray being removed by reflection. The Nicol prism, which is a composite of two properly cut and oriented calcite crystals, is joined by Canada balsam, a viscous liquid of index of refraction intermediate between n_0 and n_e for the crystal. If ultraviolet light is to be polarized by the Nicol the Canada balsam should be removed since it is ultraviolet absorbing and replaced with some non-absorbing liquid such as glycerol. With this precaution Nicol's prism is effective for producing polarized light in the ultraviolet region of the spectrum but it cannot be used in the infrared region at wave lengths greater than 2μ where the calcium carbonate absorbs strongly. Double-image prisms such as the Wollaston prism are constructed from calcite to give widely divergent ordinary and extraordinary rays so that the two beams may be compared side by side.

4. Selective Absorption

Certain colored crystals have the property of absorbing the extraordinary or the ordinary ray by different amounts. Thus a piece of the mineral tourmaline, 1 mm or so in thickness, transmits only the extraordinary ray because of the complete absorption of the ordinary ray. This crystal is not suitable as a polarizer since the selective absorption is highly dependent on wave length. A more suitable crystal is herapathite, strychnine sulphate periodide. When small flat crystals of this substance are imbedded in a plastic sheet and their optical axes rendered parallel, the matrix becomes a highly dichroic filter, Polaroid, which allows only one component to pass through over the entire visible spectrum. Polaroids have been further improved in recent years (Land and West, 1946). For example, one procedure is to subject sheets of polyvinyl alcohol which had been complexed with iodine to high stress.

The principle advantage of Polaroid over Nicol's prism is that it provides a means of obtaining wide beams of plane polarized light at comparatively small cost. In nearly all of the visible region Polaroids are as satisfactory as Nicols (Hallimond, 1944) but they are poorly dichroic in the extreme violet and red ends of the spectrum. Incidentally, because of this property two Polaroids in the crossed position can be used to remove visible light and allow only near infra-red to pass. Dichroic filters for the ultraviolet region have been suggested (Barer, 1949) but it has been the author's experience that calcite prisms are more suitable for this purpose.

III. Origin of Birefringence and of Dichroism

Anisotropy in optical properties usually goes hand-in-hand with other physical properties. For example, a birefringent crystal will have two different diamagnetic susceptibilities; a birefringent fiber will swell to a different degree transversely than lengthwise, etc. The birefringence may arise from three causes 1) the anisotropy of the orientation of atoms or of groups of atoms and the anisotropy of the chemical bonds (intrinsic birefringence), 2) the anisotropy produced by mechanical strain (photoelasticity), and 3) orientation of nonspherical particles which may themselves be isotropic and which are imbedded in a medium of different refractive index (textural birefringence).

1. Intrinsic Birefringence

Anisotropy of chemical bonds is very pronounced. For example, the polarizability (the ease with which the electron clouds can be distorted in an electric field) for the C—H bond in hydrocarbons is greater along the bond than perpendicular to it whereas with the C≡H bond in acetylene the polarizability is greater perpendicular to the bond. Anisotropy in the polarizability in chemical bonds is determined from the depolarization of scattered light as described earlier (Oster, 1948). The arrangement of anisotropic molecules or ions in a crystal lattice together with the effects of their mutual induced polarizabilities will determine the overall birefringence of the crystal (Bragg, 1924).

2. Strain Birefringence

Strain birefringence arises from the anisotropy in polarizability in what may otherwise be an isotropic material which is brought about by the application of a tension or a pressure. The application of a stress in an isotropic substance such as a glass rod can distort the electron cloud of the atoms. This is the basis for the technique known as photoelasticity which has been developed to a high degree by engineers concerned with the distributions of stress in elastic bodies (Frocht, 1941). For an isotropic body to which a stress is applied, the birefringence is positive ($n_e - n_0 > 0$) in the direction in which a tension is being applied and negative ($n_e - n_0 < 0$) in the direction in which a pressure is being applied. Strain birefringence decreases with time after the release of the strain. The birefringence and, therefore, the strain decreases exponentially with time; the time it takes to reach $1/e^{\text{th}}$ of its value is referred to as the relaxation time. In general the relaxation time decreases markedly with increase in temperature, thus one removes strains in glass by heating the glass so that there is more rapid stress relaxation. For strained gels such as gelatine which has been rapidly dried down, the stress is released if the gel is allowed to swell by the application of water. Rubber consists of coiled macromolecules which are uncoiled and oriented when the sample is subjected to stress and the sample shows an increase in birefringence (Trealor, 1949).

3. Textural Birefringence

Composite structures made up of elongated particles which are oriented or flat thin discs which are stacked upon one another as one stacks coins will exhibit a birefringence referred to as form or textural birefringence. It is not necessary that the particles be intrinsically birefringent in order that the system as a whole exhibit birefringence. Two extreme cases have been considered by Wiener (1912), namely a) when thin rods are parallel to the long axis (fiber axis) of the composite body, and b) when the thin discs are perpendicular to the long axis of the composite body (Fig. 2). If the thickness of the objects and the distance between them is assumed to be small compared with the wave length of light then the relations between the index of refraction parallel to the fiber axis $n_{\parallel}$ and the refractive index perpendicular to the fiber $n_{\perp}$ may be readily derived. However, despite the importance of these formulae a complete derivation has not heretofore appeared. The derivation is particularly simple in the case of the array of discs (Fig. 2, B). When the composite body is viewed with light vibrating perpendicular to the fiber axis there is an electric field applied perpendicular to the fiber axis. The body acts like a system of electrical condensors in

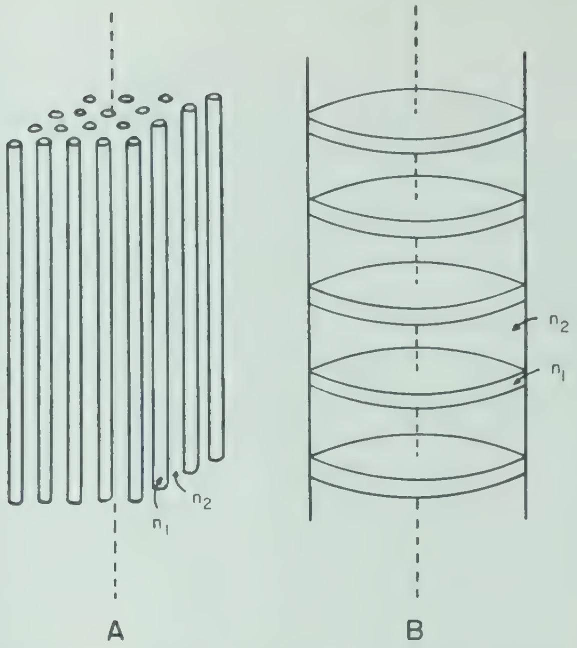

FIG. 2. Composite systems of rods (A) and discs (B). The dotted line indicates the direction of the fiber axis.

parallel and the dielectric constant $\epsilon_\perp$ in this direction (the direction of the ordinary ray) is given by

$$\epsilon_\perp = \epsilon_1 v_1 + \epsilon_2 v_2 \tag{1}$$

where the optical dielectric constants are the squares of the indices of refraction of the discs (1) and the medium (2) of volume fractions v_1 and v_2, respectively ($v_1 + v_2 = 1$). The volume fractions are proportional to the relative thicknesses of the discs of material and the medium since the body is assumed to have a uniform cross-section. When the composite body is viewed with light vibrating parallel to the fiber axis the body acts like a system of electrical condensors in series and the dielectric constant $\epsilon_\parallel$ in the direction of the fiber axis (the direction of the extraordinary ray) is given by

$$\frac{1}{\epsilon_\parallel} = \frac{v_1}{\epsilon_1} + \frac{v_2}{\epsilon_2} \tag{2}$$

Combining Eq. 1 and 2 and replacing the optical dielectric constants by the squares of the refractive indices we obtain

$$n_\parallel^2 - n_\perp^2 = -\frac{v_1 v_2 (n_1^2 - n_2^2)^2}{v_1 n_1^2 + v_2 n_2^2} \tag{3}$$

Thus the birefringence $n_\parallel - n_\perp$ for this body is always negative since the term on the right is a minus sign times a squared quantity.

The calculations for the case of the parallel rods (Fig. 2, A) are slightly more elaborate. When the composite body is viewed with light vibrating along the fiber axis, the body behaves like a system of condensors in parallel so that

$$\epsilon_{\parallel} = \epsilon_1 v_1 + \epsilon_2 v_2 \tag{4}$$

The volume fractions are proportional to the cross-sections of the rods and of the medium respectively. When the composite body is viewed with light vibrating perpendicular to the fiber axis the body acts like a complicated system of the medium condensors in parallel and in series with the rod condensor. The arrangement of the condensors depends upon the details of the model chosen. For a system similar to that shown in Fig. 2, A, Wiener calculates

$$\epsilon \quad = \epsilon_2 \frac{(v + 1)\epsilon_1 + v_2\epsilon_2}{(v + 1)\epsilon_2 + v_2\epsilon_1} \tag{5}$$

Combining Eq. 4 and 5 and replacing the optical dielectric constants by the squares refractive indices we obtain

$$n_{\parallel}^{2} - n_{\perp}^{2} = \frac{v_1 v_2 (n_2^{2} - n_2^{2})^2}{(v_1 + 1)n_2^2 + v_2 n_1^2} \tag{6}$$

Thus the the birefringence $n_{\parallel} - n_{\perp}$ for this body is always positive.

In the special case in which the index of refraction of the medium n_2 equals that of the particles n_1 then the textural birefringence is zero. This provides a convenient method for the determination of the refractive index of the particles in a composite body (Frey, 1926). If no unique medium can be found in which the birefringence is zero it may be concluded that the particles are intrinsically birefringent. This appears to be the case for myosin threads in the stretched state (Noll and Weber, 1934; Weber and Portzehl, 1952). Muscle fibers exhibit isotropic bands and anisotropic bands. The latter are believed to be due to oriented rod-shaped molecules but the connection between the optical data with that obtained from electron micrographs of muscle fibrils is not entirely clear (for references, see Mommaerts, 1950; Weber and Portzehl, 1952; Huxley and Hanson, 1954).

Most fibrous structures exhibit positive birefringence due to orientation of the long axes of the molecules in the direction of the fiber axis. For regenerated cellulose fibers the birefringence is greater the greater is the stretch which was applied during their manufacture and this is directly proportional to the tensile strength of the fiber (c.f. Meredith, 1944–1945). These fibers also exhibit an intrinsic birefringence which has been determined by the immersion technique (Hermans, 1946).

Comparatively few fibers exhibit a negative birefringence. Cellulose nitrate and polyacrylonitrile (Orlon) fibers exhibit negative birefringence due to their highly polarizable side groups which occur off the polymer chain. This effect is even more pronounced in oriented fibers of desoxyribose nucleic acid where the purines and pyrimide groups regarded as discs are lying with their flat planes perpendicular to the long axis of the molecule which coincides with the fiber axis of the sample (Signer, Caspersson, and Hammarsten, 1938). Slightly moistened fibers of nucleic acids which are subjected to considerable stretch exhibit a necking-down, the necking-down portion being positively birefringent (Wilkins, Gosling, and Seeds, 1951; Rich and Watson, 1954). Since the necked-down portion is less moist and less extensible than the other portion of the fiber, the reversal in sign of the birefringence may be due to the increased strain in the necked-down portion rather than due to some molecular unfolding mechanism which the authors proposed. Chloroplasts exhibit a negative birefringence which can be explained in terms of a lamilar structure (Menke, 1938).

Helical structures such as occur in the cell wall of cotton fibers are occasionally encountered. If n_γ is the refractive index along the elementary fibril and θ is the pitch of the helix then the birefringence may be calculated from the formula

$$\frac{1}{n_{\parallel}^{2}} + \frac{\sin^2 \theta}{n_{\perp}^{2}} = \frac{\cos^2 \theta}{n_{\gamma}^{2}} \tag{7}$$

Helices made up of straight rod elements are encountered in the inclusion bodies of plants infected with tobacco mosaic virus (Wilkins *et al.*, 1950). Composite structures encountered in myelin sheath of nerves consist of protein material oriented along the fiber axis and lipid material oriented radially outward (Bear and Schmitt, 1936). The positive birefringence is enhanced when the lipid material is extracted by solvents. Radial structures are also encountered in starch grains and in plant cell walls (Frey-Wyssling, 1930, 1940).

When a solution of elongated particles is flowed through a tube or subjected to a shear gradient between coaxial cylinders, the solution will exhibit a birefringence due to the preferred orientation of particles. The magnitude of the birefringence for given shear gradient is related to the size and shape of the molecules and to their intrinsic optical anisotropy if it is present. Spherical particles do not show flow birefringence unless they are distorted by the shear gradient. In general, the birefringence is greater the greater is the axial ratio and/or the size of the particle. These quantities in turn are related to the rotational diffusion constants of the molecules. The theory and practice of flow birefringence has been developed to a considerable extent (for reviews, see Edsall, 1942; Cerf and Scheraga, 1952). Elongated particles can also be oriented with an electric field (the Kerr effect) and from a knowledge of the time decay of the birefringence one has a means of determining rotational diffusion constants (Benoit, 1949). Flow birefringence is demonstrated very easily with freshly prepared natural actomyosin. On addition of adenosine triphosphate the flow birefringence drops markedly indicating drastic changes in shape of the molecules Dainty *et al.*, (1944). Solutions of tobacco mosaic virus exhibit strong flow birefringence which is decreased when the rod-shaped virus particles are broken down by sonic irradiation (Oster, 1947). The birefringence of tobacco mosaic virus solutions disappears when the refractive index of the medium matches that of the virus and it may be concluded, therefore, that the particles are not intrinsically birefringent (Lauffer, 1938).

4. Dichroism

The arguments given above are applicable to colored composite bodies· If the rods or discs have a light absorption coefficient different from that of the medium the body will exhibit textural dichroism (Wiener, 1926). In electromagnetic theory the absorption coefficient is proportional to the imaginary part of the refractive index. Thus one obtains formulae for the two components of absorption analogous to those obtained above for the two refractive indices.

Dichroism arises most commonly in structures which contain chemical groups which absorb light anisotropically. Substances which absorb visible light contain conjugated double bond systems such as —C═C—C═. Light with vibration along a conjugated chain or in the plane of an aromatic ring structure will be absorbed whereas light vibrating perpendicular to this direction will not be appreciable absorbed. A cellulose fiber dyed with congo red will show very strong absorption of light (green) if the plane of vibration of the light is along the fiber axis but only very little if the plane of vibration is perpendicular. It may therefore be inferred that the long dye molecules are lying along the fiber axis. The dichroic ratio, which is the ratio of the two absorption coefficients, is greater the greater the degree of orientation of the fibrillar molecules in the body. Metallic dying has been carried out with plant structures by impregnating with silver nitrate and then exposing to light to produce colloidal silver (Frey-Wyssling, 1937) but systematic studies of the dichroism of dyed biological structures have not as yet been carried out. Incidentally, certain dyes such as the azoic type attach themselves to fibers with their conjugated groupings lying perpendicular to the fiber axis so that the stained body exhibits a dichroism of negative sign.

For structures which contain natural pigments, the dichroism can be observed directly without staining. Dichroism is very feeble in chloroplasts (Menke, 1938) but in crystals of hemoglobin it is readily observable especially when blue light is used. Along a crystallographic axis (the *b*-axis) of crystalline horse methaemoglobin the absorption of blue light is more than twice that perpendicular to this axis (Perutz, Jope, and Barer, 1950). It may be concluded, therefore, that the flat surfaces of the haem groups are lying along the *b*-axis of the crystal.

The eye of the honey bee probably contains three dichroic structures and this would account for the remarkable sensitivity of the bee to polarized light such as that from the sky (von Frisch, 1950). Similarly, the eye of the hermit crab contains dichroic structues and this enables the crab to determine its position relative to the surface of the water which produces polarized light by reflection (Kerz, 1950). The human eye also contains a dichroic structure. If the sky is observed with a polarizer a yellow fuzzy elongated object is seen (two arms of a Maltese cross—"Haidinger's brushes"). The author has found, by observations through a polarizer of an illuminated ground-glass diffusing screen (a depolarizer), that the brushes rotate with the rotation of the polarizer and that the long axis of the object lies perpendicular to the direction of vibration of the polarizer. He further found that the spectral curve of the yellow brushes has a maximum at 450 mμ. It therefore follows that in the neighborhood of the fovea of the human eye there are radially arranged elongated bodies (rosettes) containing some yellow pigment whose chromophoric groups are lying perpendicular to the elongated bodies. The object appears at the "near point" of the eye (15 cm for the author) to be about 1.0 cm in largest dimension. It then follows from elementary geometrical optics of the eye that the object is roughly 0.11 cm, i.e., about the diameter of the central fovea.

Oriented fibers of desoxyribose nucleic acid exhibit a negative dichroism in the ultraviolet region, the dichroic ratio being nearly two at 260 mμ (Caspersson, 1940; Seeds and Wilkins, 1950). The negative dichroism arises because the planes of the ultraviolet absorbing purines and pyrimidines are lying perpendicular to the fiber axis. Oriented films and streams of the rod-shaped tobacco mosaic virus exhibit ultraviolet dichroism due to the absorption by tryptophane and by nucleic acid. Earlier work with this virus Butenandt *et al.* (1942) has been criticized both with respect to the observation of the sign of the dichroism and to the explanation of the origin of the dichroism Wilkins *et al.* (1950). For tobacco mosaic virus the dichroic ratio is so close to unity that the dichroism could arise from textural effects rather than intrinsic anisotropy. Dichroism studied as a function of concentration of solvent (i.e., in different stages of drying) would help resolve this question.

Techniques of measuring dichroism in the infrared region have been applied to oriented films of proteins and synthetic polypeptides (see Chapter 5 of this volume). By a study of the dichroism of the N—H bond absorption at 6.2 μ it has been established that this bond lies along the fiber axis of the molecule when the protein is in the folded (α) configuration and perpendicular to the fiber axis when the protein is in the unfolded (β) configuration in agreement with current ideas concerning protein structures (Elliott and Ambrose, 1950).

Fluorescence emitted by dye molecules which are oriented is highly polarized because of the dichroic nature of the molecules. If, however, the molecule rotates in the period between absorption and emission (of the order of 10^{-8} sec) the fluorescent light is not completely polarized, i.e., it is depolarized. The depolarization is related to the rotational diffusion constant of the dye molecule (Lewschin, 1924; Perrin, 1926). The fluorescent dye may be incorporated into a protein molecule so that the rotational diffusion constant of the protein molecule should be obtainable from the depolarization of the fluorescence (Weber, 1952). For review see Weber (1953).

IV. Experimental Techniques

1. The Polarizing Microscope

A wide variety of polarizing microscopes are commercially available. Earlier models were expensive primarily because they employed Nicol's prisms. Later models utilizing Polaroids are less expensive. An ordinary microscope can be converted into a polarizing microscope without too much trouble (West, 1942). The essential components of a polarizing microscope for studying biological structures are: a) a polarizer, b) a rotating stage, c) an analyzer crossed with respect to the polarizer, and d) a slot for insertion of a compensator (see below) at 45° to the planes of vibration of the analyzer and of the polarizer.

For ordinary purposes the light beam passing through the sample should be approximately parallel (orthoscopic observations). This is achieved by using the flat side of the microscope mirror and racking down the condensor lens (preferably of long focal length). The polarizer is placed somewhere below the sample and its plane of vibration is determined by the reflection method described earlier. The stage should be centered by the two centering screws so that sample remains exactly at the center of the field (as seen in the cross-hairs of the eyepiece) during a complete rotation of the stage. Centering is particularly difficult to achieve when a high-powered objective is used and some petrographic microscopes have provisions whereby both the polarizer and the analyzer may be rotated in conjunction, the sample remaining fixed. In this way centering is obviated.

The compensator and the analyzer are placed somewhere above the objective. In most polarizing microscopes provisions are made for these elements in the tube body. Cap analyzers with a slot for the compensator may be purchased. This is placed over the eyepiece and thereby converts an ordinary microscope into a polarizing microscope suitable for most observations of birefringent materials.

2. EXTINCTION

When an isotropic material is placed on the stage the field remains dark regardless of the orientation of the sample. If the sample is a crystalline powder one can say immediately that the material is of the cubic system. If the index of the refraction of the crystals is determined by observing the focusing effect in a number of liquids of known refractive indices (the Becke test, see for example, Chamot and Mason, 1938, Chapter 11), then the material can usually be identified from a comparison with tables (see below).

A uniaxial material, that is, one possessing two indices of refraction will exhibit a different appearance in the polarizing microscope. If one of the axes of the sample, say the extraordinary direction, is lying along the plane of vibration of the polarizer, then the extraordinary ray which is formed passes on to the analyzer where it is rejected since no component of the ordinary ray was formed so that the crystal appears dark against the dark background. If the other axis of the crystal is lying in the plane of the polarizer, the ordinary ray which is formed is rejected by the analyzer and the crystal again appears dark. Hence for every 90° of rotation of the axis of the crystal will be extinguished (parallel extinction). At all angles other than 90° the crystal will appear bright against the dark background since the plane polarized light passing through the crystal will be resolved into two unequal components and the analyzer resolves both rays into a resultant beam vibrating in the plane of vibration of the analyzer. Simple vectorial considerations show that the lighting up will be greatest when the axes of the crystal are lying 45° to the planes of vibration of the polarizer and of the analyzer. Thus extinction occurs when the vibration directions in the uniaxial object coincide with the vibration directions of the polarizer and of the analyzer.

If observations are made straight down through the optic axis of a uniaxial system (for fibers, the "fiber axis"), the object appears dark for all rotations. That is, from this direction of observation the material is isotropic. Helical structures exhibit alternating dark and light in the polarizing microscope. The dark portions correspond to the parts where the rung of the helix is pointing directly toward the observer (i.e. normal to the plane of the stage). If thin needle-like uniaxial crystals are arranged on the stage in the form of rosettes (spherulites) the sample exhibits a dark cross. The arms of the cross coincide with the planes of vibration of the polarizer and the analyzer where the crystals happen to be lying along these directions. The other crystals are not lying along these directions and hence light up. In the quantitative measurement of flow birefringence where the solution is placed between a stationary and a rotating cylinder a dark cross is observed under cross polaroids. Here the arms of the cross do not coincide with the planes of vibration of the polarizer and the analyzer but rather is rotated by an angle (the angle of isocline) which is 90° removed from the direction of the streamlines. At very low velocity gradients the most favored orientation of the elongated particles is 45° to the streamlines and the angle of isocline is zero. With higher shear gradients, however, the angle increases and its dependence on shear gradient is related to the rotational diffusion constant of the particles and hence to their size and shape (Edsall, 1942; Cerf and Scheraga, 1952).

3. Sign and Magnitude of the Birefringence

According to our definition of the birefringence, if a system is positively birefringent the velocity of the ordinary ray will be greater than that of the extraordinary ray since the velocities of the rays are inversely proportional to their refractive indices in the uniaxial body. The converse is the case for a negatively birefringent system. When light from the polarizer enters a uniaxial crystal which is not at extinction, it is resolved into an ordinary and an extraordinary ray, one of which will be the slow ray. As these two rays go through the crystal, the slow ray lags behind the fast ray, i.e., it is retarded. Suppose that at the point of emergence from the crystal, the slow ray may lag one wave length of, say, red light behind the fast ray. The analyzer resolves the two rays on to the same plane so that they are able to interfere with each other. Furthermore, the analyzer increases the phase difference by one-half wave length since the planes of vibration of the analyzer and of the polarizer are mutually perpendicular. As a result of the destructive interference red light is removed and the crystal appears green. If the phase difference due to retardation were one wave length of blue light instead of red, then blue would be eliminated and the crystal would appear yellow. If, on the other hand, the phase is one-half wave length or a fractional multiple of the wave length, no interference will take place. For a given thickness of a particular crystal, only one particular wave length of light will be cut out by interference; for other wave lengths there will be only a decrease in intensity. With increasing thickness of a particular material the colors which are observed are given in the order of Newton's scale (Table I), i.e., in the same order as that of the interference colors given by very thin films such as oil films (c.f. Rothen in Vol. II).

For most biological systems the birefringence $n_e - n_0$ may be of the order of 10^{-2} and if the thickness of the sample is 10 μ, the optical path difference is only 100 mμ. However, for destructive interference the retardation must

TABLE I

Retardation and Resulting Colors for White Illumination

Retardation ($m\mu$)	Resulting color	Order
0	Black	First
100	Iron gray	
200	Gray	
300	White	
400	Yellow	
500	Orange red	
600	Violet	Second
700	Green	
800	Yellow green	
900	Yellow orange	
1000	Orange red	
1100	Violet red	

be about five times this value. As a result the sample when illuminated with white light will not appear colored but merely grayish. If the thickness of the sample were increased five-fold, however, the sample when illuminated with white light would appear reddish since green light is removed by destructive interference. In general, it may not be convenient to increase the thickness of the sample but if we superpose a crystal having, say, a first order red tint upon the sample giving a first order gray so that their slow ray vibrations (directions of higher index of refraction) are parallel, then the resulting interference color will be of a higher order, namely blue. Thus reinforcement of interference colors indicates that the orientation of the slow ray of the sample is similar to that of the slow ray of the superposed crystal. Similarly if the slow ray of the sample is vibrating at right angles to the slow ray of the superposed crystal, then the retardations will cancel each other with a consequent decrease in the order of the interference color.

The above discussion leads logically to the conclusion that if we have a crystal plate, the compensator, upon which the direction of the slow ray is marked then by superposing this plate upon the sample under examination, we can determine from the change in interference colors the direction of the vibration of the slow ray in the sample and hence the sign of its birefringence.

Specifically, the procedure for determining the sign of the birefringence of a given uniaxial material is as follows. The sample is placed on the stage and is viewed between the polarizer and the analyzer in the crossed positions. The sample is now rotated until it is extinguished. For a fiber this will usually be along the fiber axis or perpendicular to it. The sample is now rotated 45° from the extinction position. The compensator (one of the types described below) is now interposed somewhere between the polarizer and

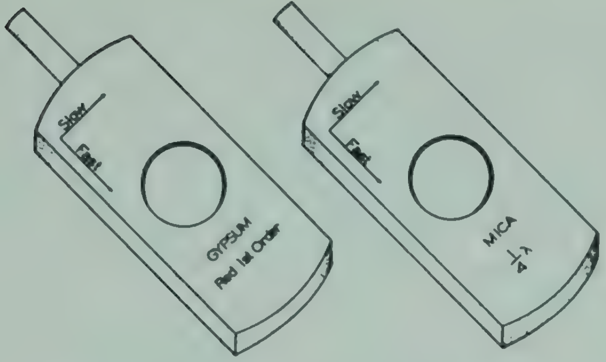

FIG. 3. Two types of compensators (from Wahlstrom, 1950).

the analyzer at an angle of 45° to their planes of polarization. There is an enhancement of interference colors if the slow direction of compensator is lying along the direction of the sample having the higher refractive index and there is a decrease in colors if the sample is rotated 90° from this position. Thus for a positively birefringent fiber, there is an enhancement of colors when the slow ray of the compensator is lying along the fiber axis and there is a decrease in colors when it lies perpendicular to the fiber axis. If the reverse situation is true, then the fiber is negatively birefringent.

Several types of compensators are available. For samples exhibiting very low birefringence and/or low thickness, the quarter-wave mica plate is useful. This compensator exhibits a first-order gray color. The selenite or gypsum plate exhibits a first-order red. These compensators are commercially obtainable where the crystal is mounted in a holder (Fig. 3). The quartz wedge is, however, more versatile. This compensator consists of a crystal of quartz cut in the form of a wedge in which the fast (lower index of refraction) direction usually lies along the long axis of the wedge. The direction of the fast or slow ray is indicated by the manufacturer. The thinnest portion of the wedge corresponds to first order gray and the thickest portion corresponds to the beginning of the fourth order colors. Thus one chooses empirically that portion of the wedge which gives the most striking differences in colors when the sample is in the two right-angle orientations. If monochromatic light is employed, the wedge exhibits a series of dark interference bands where the retardations are an integral number of wave lengths (Fig. 4).

The magnitude of the birefringence of the sample can be estimated if its thickness is known. Charts have been prepared relating the interference colors, the birefringence, and the thickness of the sample; the product of the latter two being the optical path difference or retardation. Most text books of optical crystallography contain such a chart (see, for example, Hartshorne and Stuart, 1950). In Table I are given the resultant colors for various values of the retardation. Suppose that a quartz wedge is properly placed over a sample of 30 microns thickness ($t = 3 \times 10^{-3}$ cm) to give a first order yellow (retardation 350 mμ = 3.5×10^{-5} cm). If the sample is rotated 90° and now the color is second order blue (retardation 650 mμ = 6.5×10^{-5} cm), then the birefringence which is the difference in retardation divided by the thickness is $(6.5 - 3.5) \times 10^{-5}/3 \times 10^{-3} = 0.01$. Conversely, if we knew the birefringence of the sample we could estimate its thickness by use of the quartz wedge.

More elaborate compensators involving movable quartz wedges and mica plates have been devised to accurately measure the retardation. The theory of such compensators has been developed in detail (Jerrard, 1948). One of these, the Babinet compensator, consists of two wedges, one cut with its slow ray along the wedge and the other with its fast ray along the wedge. One wedge is moved with respect to the other by means of a screw. At some position the thickness of the wedges are identical so that the birefringence

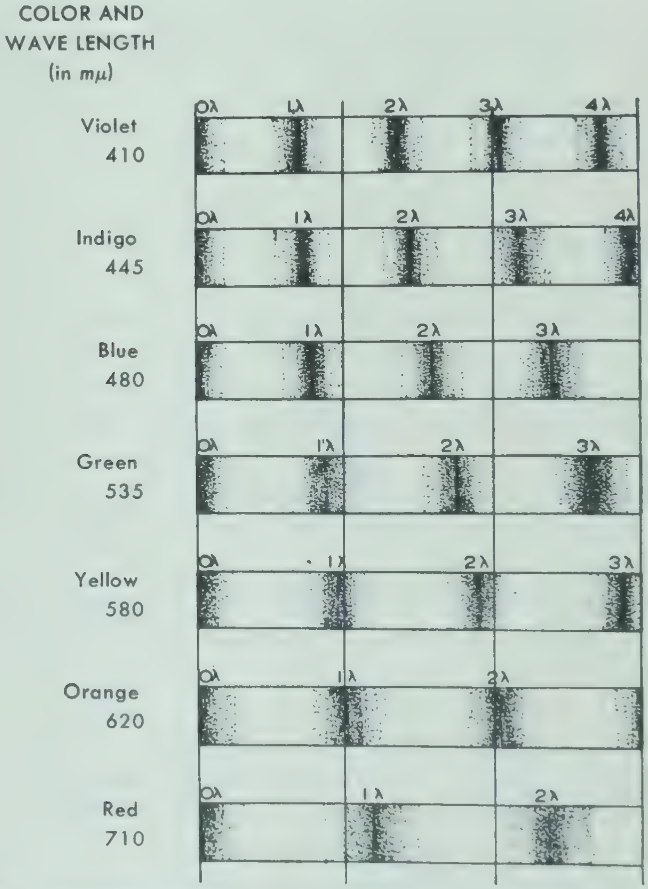

FIG. 4. Interference for a quartz wedge (from Wahlstrom, 1950)

of one wedge is exactly cancelled by that of the other wedge and the effect is that of a plate of zero thickness. On either side of this position there will be a positive or a negative relative retardation. Hence a series of colored bands will appear about the center gray band where the relative retardation is some integral multiple of the wave length, the relative retardation being read directly from a calibrated scale attached to the screw. The compensator is placed at 45° between the crossed polarizer and analyzer. The birefringent sample with its optic axis at 45° is introduced and causes a displacement of the bands. The screw is then turned until the band which originally coincided with the cross-hair is returned to its former position and the retardation is read off the scale. This arrangement is satisfactory if the retardation is of the order of one wave length. For smaller values of the retardation the compensator should be rotated to within a small angle of the plane of vibration of the polarizer. The illumination is considerably decreased but the retardation of weakly birefringent materials can be measured in this way (Ferry, 1941).

Measurements of extremely small retardations of less than 5 mμ pose several difficult experimental problems. It is essential that the lenses of the microscope be strain-free, unless the strained glass itself is used as the compensator. Polarization due to the scattering and reflection by the sample, the glass slides, and the lenses may obscure the birefringence of the sample. Nevertheless, by the judicious use of very thin mica sheets as compensators and the exercise of care in arranging suitable illumination, retardations as low as 0.03 mμ have been measured (Swann and Mitchison, 1950; Inoué, 1951).

Perhaps the most frequent use of birefringence techniques is in the identification of crystals in the polarizing microscope. As mentioned earlier, an isotropic crystal (a crystal of the cubic system) is identified by the determination of its single refractive index. Uniaxial crystals (crystals of the hexagonal, trigonal, and tetragonal systems) are identified by the birefringence and one index of refraction measurement together with certain observations of the geometrical appearance of the crystals. Biaxial crystals (crystals of the monoclinic and triclinic systems) are identified by their birefringence and one or more index of refraction measurements together with a determination of the angles between the extinction directions. One technique involves the use of convergent light (conoscopic observations) to give optic figures which readily aid in the identification of uniaxial and biaxial crystals. The technique of identification of crystals by optical means is a highly developed subject. The beginner is advised to first try the experiments described in Chapters 9, 10 and 11 of Chamot and Mason (1938) and then proceed on to the book of Hartshorne and Stuart (1950) where the theory of the technique is developed and a number of useful hints are given. There are a number of tables describing the optical characteristics of inorganic crystals (see, for example, Lange, 1952) and a recent book describes the optical characteristics of more than 2500 organic crystals (Winchell, 1954).

4. Measurement of Dichroism

Observations of dichroism are made only with one polarizer since the sample itself acts as a polarizer in the spectral region where light is absorbed. The sample is placed on the microscope stage and is rotated with respect to the polarizer, the analyzer having been removed. The effect is very striking with crystalline dyestuffs. For example, in one position crystalline malachite green appears dark green but 90° to this position the crystals appear bright red. If the polarizer is removed and a calcite crystal of sufficient thickness is placed in the tube body, two images of the crystalline malachite green will appear side by side, one red and one green. Another useful test object is commercial stained Cellophane.

Even if a given pigment is not obtainable in crystalline form, its dichroic spectra can be obtained by applying the pigment to a highly oriented substrate such as ramie fiber or incorporating it into a film such as Cellophane (or in the case of ether-soluble pigments, into a film of cellulose nitrate) and stretching the film almost to the breaking point. The dried stretched film will then be dichroic with positive dichroism if the conjugated double bonds are lying in the direction of the applied stress. Such films serve as useful dichroic filters (Käsermann, 1948). When basic dyes are applied to oriented fibers of nucleic acid the bodies are dichroic (White and Elmes, 1952). Basic dyes have a very high affinity to special faces of certain inorganic crystals due to the local high density of negative charge on these faces (Anderson, 1949). The dye molecules are highly oriented and hence exhibit dichroism.

In the author's laboratory dichroic ratios are determined photoelectrically. The two images produced with the calcite prism are scanned with a photomultiplier tube and the ratio of the photocurrents produced for the two images is determined and compared with the photocurrent for the background. These measurements are carried out using various wave lengths of incident parallel light, rendered monochromatic by the use of a grating monochrometer. In order to transmit all wave lengths (from 240 mμ to 700 mμ) and to have the image always in focus, the ordinary glass lenses of the microscope are replaced by a reflecting condensor and a reflecting objective and a quartz eyepiece is employed. The sample is placed on or between fused quartz slips. An alternative arrangement has been employed by Seeds and Wilkins (1950) who analyze the resultant spectra of a single image of the sample using a Wollaston prism as the polarizer. A review of the various methods of measuring dichroism in the ultraviolet region of the spectrum has recently appeared (Seeds, 1953).

The dichroic ratio of most substances which have been examined is usually quite small, i.e., close to unity, although dyed ramie fibers may exhibit a dichroic ratio of nearly ten (Preston, 1931). It is important to realize that the dichroic ratio of a given material is strongly dependent on the wave length of the light since the dichroic ratio is the ratio of the optical densities of the two principal directions in the sample. As with the usual birefringence measurements, dichroism should be observed with parallel light since the dichroic colors vary with the direction of the rays in the sample.

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Chapter 9

ELECTRON MICROSCOPY

V. E. Cosslett

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I. The Limits of Optical Observation

1. INTRODUCTION

The electron microscope is primarily useful because it possesses a higher resolving power than that of any system employing light as illuminant. Therefore it is necessary first to discuss the limits imposed on direct observation, either with the unaided eye or through an optical instrument, by the nature of light itself. It will appear that the best resolution attainable is about 2000 Å with visible light and 1000 Å with ultraviolet, assuming an object of high contrast. The theoretical limit with electrons is below 1⁄10 Å. Present uncorrected electron lenses have achieved 10 Å with high contrast specimens, but only 20–30 Å with biological material. For comparison, the spacing of atoms in molecules or in crystals ranges from 1 to 5 Å. The high resolution of the electron microscope is bought at the cost of certain disabilities concerning the types of specimen which may be examined, in particular that they must be desiccated and thus dead, or at least in a state of suspended animation. These difficulties will be discussed in later sections of this Chapter.

2. RESOLVING POWER

The wave nature of light sets a lower limit to the size of object point that can be resolved, that is, clearly seen as separate from a point of the same size when the two are in contact. It is clear that contrast must be involved in any such observation, and it is assumed for further discussion that the object points are completely opaque. An exact treatment by diffraction theory shows that the angular width 2α of the beam received by the eye (or other recording system) is also involved (cf. Fig. 1). Regarding the information about the object as being conveyed in a spherical wave originating at the object, it follows that the wider the wavefront gathered by the eye, the greater is the information recorded and hence the easier it is to distinguish the point from a neighboring point. The resolving power as limited by diffraction can then be defined as

$$R_D = \lambda/N_A \,. \tag{1}$$

if λ is the wave length of the illumination and N_A is the numerical aperture of the viewing system. Account must be taken of possible differences between the refractive index of the media in which the object and image are respectively situated, since this will influence the velocity of light and hence its wave length. The full definition of numerical aperture is $N_A = \mu \sin \alpha$, where μ is the re-

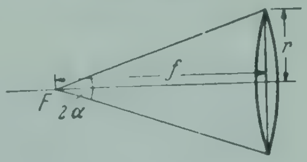


FIG. 1. Angular aperture of lens.

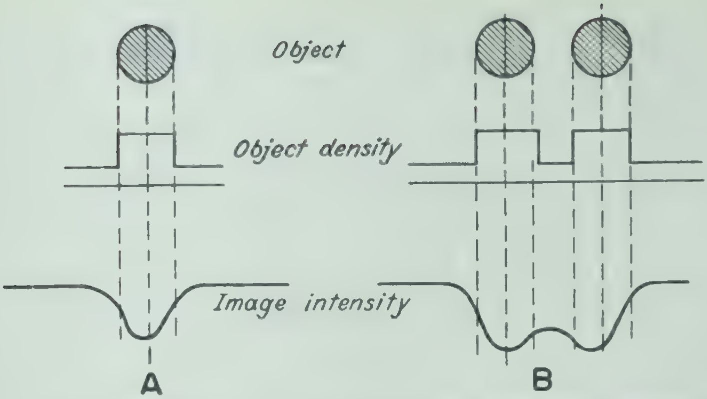


FIG. 2. Intensity distribution across image (A) of single particle, (B) of two neighboring particles.

fractive index of the object space relative to the image space. So long as the angle α is small, as it is in present electron lenses, we can approximate its sine to its tangent and write $N_A = r/f$ for objective lenses, where r is the radius of the lens employed and f its focal length.

Equation 1 is the simplest form for the resolving power, but some expressions current include a multiplying constant, the exact value of which depends on an estimation of the minimum difference in contrast that is detectable. Figure 2 shows the appearance of the images of two opaque object points, with the corresponding density graph. The minimum fall in contrast, along the line joining them, for points to be resolvable will depend on the properties of the recording system and will vary from person to person when this is the eye. It is conventional, following Abbe, to take 25 % as the minimum discrimination of the eye and this results in a slight decrease in the value of R as given by Eq. 1. For the discussion of electron microscope resolving power it is adequate to take Eq. 1 as it stands for the diffraction limitation, or rather, since $\alpha \fallingdotseq \sin \alpha$, to use the form

$$R_D = \lambda/\alpha \tag{2}$$

In the normal electron microscope both object and image are at ground potential and hence in regions of the same refractive index, electrically interpreted, so that μ does not enter into Eq. 2.

3. THE EYE AND THE MICROSCOPE

In the case of optical recording systems the numerical aperture has to be evaluated with due regard to the refractive index around object and image and to the angular aperture. When this is done for the case of the unaided eye Eq. 1 gives a resolving power of 0.1 mm, which agrees well with practical tests under optimum viewing conditions. An optical microscope, as explained in Chapter 6, is in principle simply a device for increasing the effective numerical aperture of the viewing system. The object may be brought close up to the objective lens, giving a large value of α, and N_A can be further increased by surrounding the object by oil of high refractive index. In this way the value of N_A can be made as high as 1.5 in the best case, so that the resolving power from Eq. 1. becomes less than the wave length of the light used. By using oblique illumination a further factor of up to 2 can be secured, so that the absolute limit of optical microscopy can be set as $R = \lambda/3$. With visible light of mean wave length 5400 Å, we have $R = 1800$ Å; with monochromatic u-v of 2400 Å, the theoretical limit is 800 Å. Although it is difficult to carry out tests of resolving power that accord precisely with the postulates of the theory, these values agree closely with the best results claimed in visible and u-v microscopy respectively.

It must be stressed that resolving power as thus defined involves the discrimination of two neighboring points; it does not represent the extreme limit of *detection* of isolated particles. This is a distinction which has to be borne in mind especially when evaluating the resolving power from electron micrographs, where it is rare to find pairs of points sufficiently close to apply the proper measure of R. It is much easier to determine the minimum size of particle that is visible in the image. However, the relation between this limit of detection and R depends on both the shape and the contrast of the object (Section VII, 3). For this reason, any claim to extreme resolution on the basis of sharpness of picture has to be accepted with caution.

4. ELECTRON ILLUMINATION

An electron beam may be shown, both by theory and by diffraction experiments, to have wave properties and a relation can be found between its velocity and its effective wave length. Translated into terms of the voltage (v) employed to accelerate it, the equivalent wave length of an electron beam (in Å) is given by

$$\lambda = \sqrt{\frac{150}{\mathrm{v}}} \qquad (3)$$

neglecting a relativistic correction term which becomes important only at very high voltages. It follows that the wave length of a 60 kilovolt beam is 0.05 Å, i.e., very much less than atomic diameters. Beam voltages of this order are needed in electron microscopy to give reasonable object penetration. Equation 1 shows that a resolving power 100,000 times better than that of the best optical microscope would be possible with a perfect electron microscope. In fact, as discussed in Section IV, the available aperture of present electron lenses is so small, on account of spherical aberration, that the theoretical limit is about 5 Å and the best practical value attained is around 10 Å. The optical microscope attains its theoretical limit only because means have been found of correcting the aberrations from which a simple glass lens also suffers. It is thus possible to make a compound immersion objective of $N_A = 1.5$, whereas the normal electron lens has to be stopped down to between $1/100$ and $1/1000$ numerical aperture.

Even so, the minuteness of the electron wave length allows a resolution of tens of Angstrom units to be obtained. Hence the electron microscope already largely closes the gap between the limit of optical observation and the size range of x-ray methods. In this region a great variety of biological detail occurs, whether isolated units such as viruses and macromolecules or structural units of larger organisms and bulk tissue, such as muscle and nerve fibrils, cell membranes and chromosomes. The electron microscope has already been applied to many of these specimens, and the general principles of preparing such material for inspection will be set out in later sections. First the physical principles of the electron microscope and its operation will be described, and in Section III the essential differences between optical and electron microscopy will be further discussed. It may be said at once, however, that the two techniques are complementary and should always be used to reinforce each other. It is obvious that optical control of the reliability of electron micrographs must be used as far as it will go; it is now also beginning to be appreciated that the electron microscope can repay this debt in kind, by providing a check on artefact production in many of the established staining techniques of optical microscopy.

II. Basic Principles of Electron Microscopy

1. THE LENS SYSTEM

In its general lines of construction and operation the electron microscope closely resembles the optical microscope. Each requires a source of illumination, a condenser lens for controlling it, an objective lens and one or more further lenses for providing high magnification. The main differences between the two instruments are those of detailed design, arising from the special properties of electron beams as compared with light. Some minor differences of principle arise from the same cause, e.g., the electron image is viewed on a fluorescent screen, not directly, and interchangeable lenses are not required owing to the continuously variable power of magnetic fields. Since practically all commercially available electron microscopes employ magnetic lenses, the discussion will be carried on in terms of these rather than of electrostatic lenses. The differences between the two types are in any case matters of practical details of design and operation rather than of principle or ultimate performance.

A schematic diagram of the imaging process in the electron microscope (Fig. 3), compared with an optical microscope (inverted from its normal position) makes clear the essential similarity of the two. The electron illumination is drawn from a white hot tungsten filament C by the strong electric field maintained by a negative high potential applied between C and an anode A, which is grounded. A shield electrode, at a slightly higher negative voltage than C, surrounds it and serves to increase the brilliance of the beam that emerges from the aperture in A. The whole assembly is known as the electron gun and serves to deliver an intense narrow beam into the condenser lens L_c, which transmits part of it through a limiting aperture at its center. By varying the strength of the magnetic field in L_c the angle of divergence and brightness of the illumination falling on the specimen O are controlled. The whole electron path, and therefore the specimen also, must be *in vacuo*. O is situated just before the focal plane of a magnetic objective lens L_o of short focal length, which produces an enlarged image I_1 in the focal plane of the projector lens L_p, also of high power. L_p throws a further enlarged electron image on to a fluorescent viewing screen S or on to a photographic plate which can be exposed by raising S. Focusing is carried out by varying the magnetic field in L_o, so that I_1 falls in the correct position relative to L_p. Magnification is varied by changing the strength of L_p, which has such a great depth of focus that it is unnecessary to re-adjust L_o except when the strength of L_p is greatly changed.

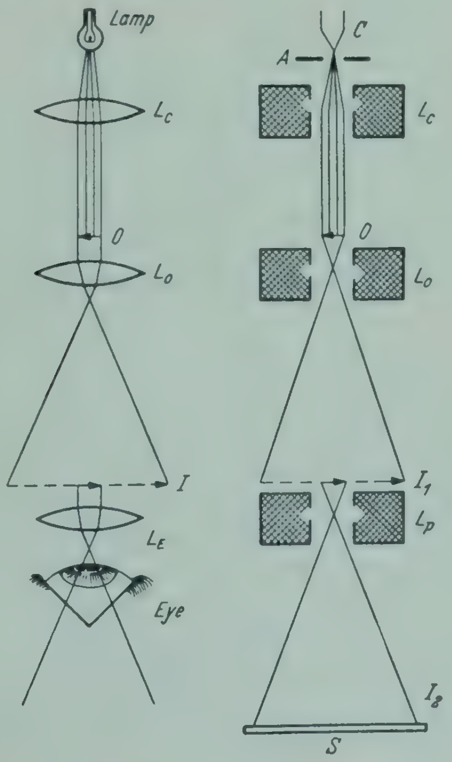


FIG. 3. Electron microscope and optical microscope: schematic diagram of image formation.

It will be seen that the operation of an electron microscope is unlike that of an optical microscope in two essential respects. Firstly the lenses are continuously variable in strength since the current flowing in the windings of the magnetic lenses can be smoothly controlled with a variable resistor. Focusing involves no mechanical motion of the lenses but only the variation of a current that changes the focal length of L_o. Secondly, the magnification is produced in roughly equal proportions by objective and projector, the latter being of high power and controlling the magnification. In practice it is found that too much distortion of the image is produced if the projector is reduced greatly in strength so that interchangeable projector polepieces are sometimes provided in magnetic microscopes having two stages of magnification. In the optical microscope magnification is usually altered by changing the objective lens, of course. Several recent electron microscopes incorporate a third stage of magnification (Fig. 13), by means of a weak intermediate lens L_i placed between L_o and L_p (cf. Section VI). In this case the intermediate lens is varied for magnification change, and L_o and L_p require only minor adjustment. As L_i can be used either to increase or reduce magnification, its combination with L_p allows a very wide range of magnification (1000 to 60,000×) to be covered without changing polepieces. The different commercial models of electron microscope on the market are described in Cosslett (1951).

2. Image Formation

The production of detail in the electron image depends on differential scattering of the illuminating beam in different parts of the specimen. Even with an accelerating voltage of 100 kv the penetrating power of the beam is so poor that only a very thin specimen—less than 0.25 micron thick for biological material—can give enough transmission to produce a visible picture. Most objects contain regions of varying thickness and these will transmit electrons to different extents, so that corresponding contrast variations are seen in the image. Scattering rather than simple absorption is the mechanism by which electrons are removed from the beam, each electron usually suffering many collisions before being finally absorbed. In the light microscope scattering is negligible and contrast is produced almost entirely by differential absorption in the object. In present electron lenses the permissible aperture is so small that even slightly scattered electrons are removed from the beam and so assist in creating contrast.

The probability of scattering depends on the amount of matter in the path of the electron, so that the contrast in the image displays directly the distribution of matter in the cross section of the object presented to the beam. "The amount of matter" here means the product of the number of atoms and their physical density or atomic number. Hence the electron image can give no information about the chemical constitution of an object unless the local thickness is known and unless it is locally homogeneous. Attempts to identify particular elements by their specific absorption of electrons have so far failed.

The electron microscope image thus gives information about the distribution of matter in the specimen, understood as mass density. It is not directly comparable with the optical image of the same region, since local absorption of light depends on chemical constitution rather than the actual amount of matter present. As is well known, the transparency of many materials (especially in biology) prevents their direct observation by normal light microscopy, for which reason various specific staining techniques have been evolved for producing visible contrast in selected constituents of a specimen. It is possible that a similar variety of selective treatments will be discovered for electron microscopy, but so far little progress has been made. The principle here is to absorb heavy elements on to specific biological structures, so that their electron scattering power is increased. So far it appears that the absorption of agents such as lead salts, osmium tetroxide or phosphotungstic acid is too general, although some success has been achieved with the last named (cf. Section X, 3.).

It should be noted that phase contrast microscopy gives a picture that resembles very closely the electron image in its contrast structure. The production of phase delay in a light beam is determined by the optical path length traveled in the specimen, and this by definition is given by the product of thickness and refractive index. Hence the contrast in a phase image is essentially dependent on the same factor as in the electron image—the amount of matter in the path of each component beam. An electron micrograph can thus be more profitably compared with a phase contrast than with a normal optical micrograph. Such comparisons indicate that the degree of distortion in the preparation of material for electron microscopy is much less than originally supposed.

III. Practical Differences Between Optical and Electron Microscopy

As indicated in the previous Section the chief operational difference between an electron and a light microscope lies in the continuous variability of the focal length of an electron lens. So the operator looking at the electron image simply turns control knobs, as on any television set, to obtain the clearest picture and also to change its magnification. This advantage in convenience is offset, however, by several limitations that are not encountered in optics. These require separate discussion; they almost all arise from the interaction of the electron beam with the specimen, as discussed above, and from the aberrations of electron lenses. Limits are set to the thickness of specimen that can be examined, conditions of high vacuum and electrical stabilization are imposed that add to the complexity of operating and servicing an electron microscope, small apertures have to be adjusted at the proper locations in the beam path, and precautions have to be taken about electrical and thermal disturbance of the specimen. It is desirable to consider at this stage the nature and operational influence of these special features of electron microscopy; their proper control will be dealt with in Section VIII in connection with the attainment of high performance.

1. Specimen Thickness

The poor penetration of electrons, even at high accelerating voltages, requires that the object examined be extremely thin. The limiting factor is the amount of energy lost by electrons through inelastic scattering in passing through the object, i.e., by collisions in which energy is transferred to the atoms struck. As a result a proportion of the electrons is slowed down, and the beam acquires a voltage spread that is equivalent to loss of monochromatism in light. As explained in Section IV, electron lenses have severe chromatic aberration which causes blurring of the image unless the beam is uniform in voltage. Hence this chromatic confusion increases with the thickness of object traversed, and in fact spoils the image well before the object is opaque to electrons.

It is found in practice that biological material can be examined up to a (dry) thickness of about 0.1 μ (1000 Å) at a voltage of 50 kv, so long as a resolution better than 100 Å is not required. At 100 kv the tolerable thickness is roughly twice this figure. As a general rule it can be taken that the specimen thickness should not be more than 10 times the required resolution; with compact material, such as metal films, it should be thinner. Hence the great depth of focus available in the electron microscope (see Section VI) cannot be usefully employed in transmission operation at high resolution. Its main advantage lies in the image space, where it allows great latitude in the positioning of the recording plate.

The extreme thinness thus demanded in the specimen is not such a great limitation as might be expected on the types of material that can be examined. Just because the electron microscope is needed to explore beyond the limits of the optical microscope, the objects to be examined will often themselves be very minute, such as bacteria, viruses, macromolecules, fibrils. The thickness of 1000 Å quoted above refers to dried material, so that bacteria which are up to 1 micron thick in the wet state still show considerable detail at 50–70 kv, unless grown on a very rich medium. But the larger microorganisms, such as spermatozoa, protozoa, spirochetes, are already too thick for internal detail to be seen, even at 100 kv. The solution is either to submit them to mild enzymatic digestion or to embed and section them, as described in Section X, 2.

2. VACUUM OPERATION

The ready scattering of electrons also requires that their path within the microscope be evacuated of air as completely as possible. A residual vacuum poorer than about 5.10^{-4}mm Hg is liable to give too much background fog in the image, due to scattering of the beam, as well as leading to electrical breakdown in the gun and shortening of the filament life. High speed pumps have to be provided to maintain the vacuum and the instrument itself has to be constructed in such a way that it is leak tight and yet easily demountable in certain parts for servicing. This is readily achieved with rubber sealing rings between stationary parts and metal bellows between moving parts, but it adds to the complexity of the apparatus and admits more possibility of breakdown. Indeed, the necessity for vacuum operation, along with that for voltage and current stabilization, provides the main reason for the high cost of an electron microscope compared with the optical instrument.

Fortunately the design and construction of vacuum systems is now so well developed that very little trouble is to be expected from an electron microscope in this respect. The parts most likely to develop a leak are naturally those most often opened, such as the object chamber, the electron gun and the camera. Simple instructions are always provided by the makers about testing for high vacuum, and on renewing rubber gaskets which show signs of wear. Similarly advice is given on the care of pumps and their regular recharging with oil. As regards routine operation, pumps of such speed are now fitted that the delay after changing the specimen or photographic plate (or film) is reduced to the order of a minute. With some emulsions, however, the pumping time may be so long that it is advisable to pre-pump them in a separate bell jar and transfer them to the microscope as rapidly as possible.

3. MONOCHROMATIC ILLUMINATION

In optical microscopy monochromatic illumination is sometimes desirable; in electron microscopy it is essential. As mentioned above, and in detail below (Section IV), the lack of correction of electron lenses makes it necessary to ensure high uniformity of velocity in the electron beam. Since the focal length of a lens is determined by the strength of its field as well as the electron velocity, it follows that the electrical supply to the lens must also be highly constant. In a normal magnetic objective lens, for instance, the energizing current must be held constant to within a few microamps in a total of 100 milliamps. A corresponding stability, better than 1 part in 10,000, has to be ensured in the high voltage supply.

In practically all modern instruments the required stabilization of both high tension and lens supplies is produced by electronic means, operating on the feed-back system by comparison of part of the output voltage with long-life reference batteries. Such equipment is again very complex and the possibility of breakdown is by no means negligible. A more important consideration, however, it that the best resolution cannot be obtained from an electron microscope unless the circuits are all tuned up to their peak of efficiency. Small faults can only too readily occur, leading to the generation of parasitic oscillations or the departure of amplifiers from their optimum conditions, which will impair the stabilization of the supplies for the microscope.

So long as a comparatively poor resolution of the order of a few hundred Angstrom units is adequate, as it is for many routine purposes, the electrical equipment can be largely left to itself apart from annual tests of components. But if a resolution of better than 100 Å is needed the circuits must be regularly checked, and if 20–40 Å is sought, then very great care must be given to stabilization. Meters are provided for measuring some of the main currents and voltages in the circuits, and usually more detailed advice is provided in the operating handbooks about the testing of output with an oscillograph. But it has to be recognized that it is impossible to keep an instrument in peak performance without the regular services of an expert in electronics. In many biological laboratories such specialists are now available. In places where they are not, it is advisable to make arrangements with the makers of the electron microscope to have it serviced regularly. Even if only modest demands are made on it, such attention is desirable; when really high resolution is consistently required, then it becomes indispensable. In optical microscopy there is little to go wrong with the instrument, and the attainment of high performance with a first-class microscope is largely a matter of skilled operation. With the electron microscope, skill in operation plays a minor part, the determining factors being the proper preparation of the specimen on the one hand, and the careful tuning-up of the machine beforehand on the other. Apart from checking of the electrical supplies, the correct alinement of the electron microscope is of the greatest importance—partly again because of the chromatic requirements—and this will be discussed in Section VIII, 5.

4. Contrast and Apertures

As mentioned in Section II, contrast in the electron image arises from differential scattering in the specimen, those electrons which are scattered through an angle of more than a few thousandths of a radian being lost from the imaging beam. They may be eliminated most simply by placing a small stop in the objective lens, the size being determined by the value of the spherical and diffraction aberrations for the lens and the resolving power desired. In a normal type of magnetic lens with a focal length of 5 mm and an optimum aperture of 6×10^{-3} radian, corresponding to a resolution of 12 Å, the aperture in the stop would be 60 microns in diameter. Metal discs with holes in this size range are provided for most electron microscopes, with a device for supporting them in the appropriate position in or near the back focal plane of the lens. In many instruments the aperture can be centered during observation of the image, which greatly facilitates the exact alignment of such a fine hole.

In principle a physical aperture is not essential for high resolution: the severe spherical aberration of the objective will have an aperturing effect and throw scattered electrons out of the imaging beam. But operation without an aperture demands very careful alignment and control of the illuminating conditions, and also results in poorer contrast in the image than when an aperture is used. It is therefore best to go to the trouble of centering an aperture, at least wherever the specimen is of poor natural contrast. Many specimens, especially when fixed with osmic acid or other heavy metals, are already of high contrast and operation without an aperture is perfectly satisfactory. The same is true of metal-shadowed preparations, provided that a metal coating tens of Ångstrom units thick can be tolerated. When details finer than 100 Å are sought, however, it is preferable to insert an aperture and use little or no shadowcasting.

Normally the aperture disc will receive heavy electron bombardment, as a result of which a semi-conducting organic deposit forms on it. It appears to be impossible to avoid such contamination, which also occurs on the specimen and indeed on any surface reached by electrons (cf. Section VIII). It comes from residual oil vapor in the column of the instrument, which cannot be removed to the necessary degree in a demountable system such as an electron microscope must be. It does not form on regions which are so heavily bombarded that the temperature rises above about 200°C (Ennos, 1953), so that the condenser lens aperture is usually not affected. All other apertures, and especially the objective aperture, have to be cleaned from time to time. The interval varies with the machine and the frequency of its use, and may be a few days or a few weeks. The symptoms are drifting of the image and pronounced astigmatism (Section VII). The instructions book should give details of how to remove and clean the aperture. If there is an aperture in the projector, as is sometimes fitted to prevent scattered electrons from reaching the image, this also must be removed and cleaned from time to time.

5. Effect of Electron Beam on Specimen

In passing through the specimen the electron beam conveys charge and thermal energy to it, as well as causing contamination to be deposited. In optical microscopy it is rare for the specimen to be in any way affected by the illumination, but in electron microscopy the first two effects mentioned are inseparable from the imaging process.

Electrostatic charging is rarely troublesome, however, since few electrons are actually absorbed in thin specimens and such charge as is received leaks away to the metal support grid very rapidly. Difficulty is occasionally found when relatively large pieces of insulating material are examined, such as inorganic crystals or glass. A heavy coating of shadowing metal on the support film often provides sufficient conduction in such cases. An alternative is to employ a low voltage electron beam for decharging, as devised for electron diffraction operation (Trillat and Oketani, 1950).

Heating of the specimen is more difficult to avoid, and may be excessive when thick specimens are used. Apart from more frequent inelastic scattering, energy is then more often delivered to the object by electrons which are completely stopped in it. It appears that specimens of ordinary thickness, under the moderate illumination required for viewing at 5,000 to 10,000 magnifications, reach a temperature of the order of 150–200°C (von Borries, 1949). By careful control of the illumination, dark adapting the eye, and especially by fitting a small aperture just above the object, it is possible to keep the temperature down to 50–60°C. Using a special protective device, von Ardenne (1948) claims to have observed crystals of sulfur at high magnification without melting them (m.p. 110°C). It is common experience that wax embedded sections can be observed satisfactorily without melting.

If trouble occurs it is simplest to try the effect of fitting a small metal washer above and in contact with the electron microscope specimen grid, as is possible in several types of specimen holder. Alternatively it may be necessary to make a special holder of high thermal capacity. Usually it is sufficient to use minimum illumination and dark-adapt the eye. For this reason it is good practice to operate at low or moderate magnification, requiring low illumination, and to employ a ×10 optical viewer for focusing the image (cf. Section VI).

Not only does the beam heat the specimen, it often produces chemical change in it. This may simply be a reduction of organic substances to carbon (König and Winkler, 1948) but may be more complex. Little systematic investigation has been made of these effects, but evidence is now coming from the use of high energy electron beams in nuclear physics and radiotherapeutics. It is highly probable that most organic tissues are quickly reduced to carbon under ordinary conditions of illumination, and some constituents can be completely evaporated (Burton *et al.*, 1947). When the object is thick and dense as in the case of inorganic crystals, it often happens that the main bulk evaporates, leaving an empty shell that has been formed by contaminant deposition.

Because of the temperature rise, and also the damaging effect of ionization, it seems improbable that living matter can be examined in the electron microscope. Even if it can survive drying, as is possible with some bacteria, it is highly probable that it will be killed by the amount of exposure to the electron beam needed for focusing and photographing (Cosslett, 1951; p. 261).

IV. Elements of Electron Lenses

1. ELECTROSTATIC LENSES

In order to operate an electron microscope it is not at all necessary to know how an electron lens focuses the beam. But, as in optical microscopy, an understanding of fundamental principles makes it easier to grasp the need for those small refinements of technique that make all the difference in getting the best possible performance from an instrument. It also helps the non-physicist to appreciate the limitations of the electron microscope. As electrostatic lenses are the simplest to understand, and enter into the electron gun if not into the imaging system, they will be described first; the mode of action of magnetic lenses is more complicated, as the image is rotated as well as focused.

The analogy between gravity and electrical potential helps in getting a picture of how electrons are deviated in electrostatic lenses, which in essence are nothing more than properly dimensioned metal tubes or discs (electrodes) maintained at different voltages. Just as material particles move downhill from a higher to a lower level under the attraction of gravity, if unconstrained, so do charged particles move towards an electrode carrying charge of opposite sign. Consider a simplified model of an electron gun. As electrons are negatively charged, the cathode is represented as at high negative potential and the anode at zero (ground). The latter necessarily has an aperture and it is usual to place a rather smaller aperture just before the cathode, in the shield *S* (Fig. 4). If a rubber sheet be imagined pinned down along the cathode edge, supposing it to be a flat surface, and beyond the anode face, the conformation shown in Fig. 4 will result. There will be a trough in the shield aperture and a ridge in the anode aperture. It can be realized at once that particles emitted from a cathode point *F* on the axis of the system will run together in the trough and be spread out in passing through the anode. In such simple electrode systems, an aperture in an electrode of the same sign as the particle will have a focusing action and one

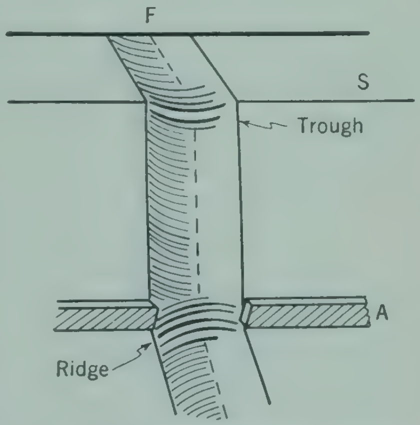


FIG. 4. Gravitational model of electrostatic field between a plane cathode *F*, shield *S* and plane anode *A*.

in an electrode of opposite sign has a defocusing or diverging action. The relative strengths of these effects depends on the potential applied to the electrodes and on the speed of the particle passing through them. In the gun, the electrons are emitted from *F* at low velocity so that they are sharply focused by quite a small voltage on the shield or even by zero voltage; this means that the bias voltage on an electron microscope gun is a very sensitive control of illumination (cf. Section V). But at the anode the particles are at the "foot" of the slope and have such high velocity that the diverging effect is small. Usually, however, they have been so strongly acted upon at *S* that they are already diverging when they reach *A*, as indicated in Fig. 3. It is possible to design the electron gun so that the combined action of *S* and *A* actually gives a convergent beam at a spot beyond *A*, where the specimen will be placed. Such a system is used in the French CSF (Bricka and Bruck, 1948) and in the German AEG (Steigerwald 1949) electrostatic microscopes, but all magnetic microscopes use guns that produce diverging beams. A condenser lens is then used to focus the illumination on to the specimen, as described in the next section.

It is clear that the combination of *S* and *A* will have some sort of focusing action no matter what the point of origin of the electron beam, i.e., *F* need not be close to *S*. If it is in fact a gun at some distance delivering a beam into a system of two apertures at different voltages, these will focus the beam to an extent that will depend on their potential difference and spacing. That is to say, such a pair of apertures forms the simplest type of electron lens. A more common type, as used in television tubes, consists of two coaxial cylinders with a small gap between their ends and at different voltages (Fig. 6). The form of the field is much as in Fig. 4, but the trough now merges rapidly into the ridge within the second tube. As the electrons will still be traveling faster over the latter than through the trough in the first tube, the overall focusing action is always positive. In fact, only a simple aperture can have a net diverging action on electrons.

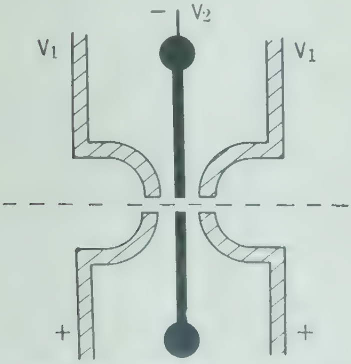


FIG. 5. Electrostatic unipotential lens.

The type of lens usually used in electrostatic electron microscopes is shown in Fig. 5, consisting of three apertures, the outer two being at common voltage (ground) and the center one connected to the cathode supply. Such a unipotential lens, using only a single voltage, has a very short focal length. It might seem that electrons would not pass the central aperture, as it is at the same potential as the cathode, but in fact there is a "trough" at its center owing to the effect of the neighboring electrodes—just as there is in *S* in Fig. 4 even if *S* is at the same voltage as F. Hence there is a slowing up of electrons in the unipotential lens, with consequent strong focusing action. If a still higher negative potential is applied to it, then electrons cannot pass and it functions as a mirror.

2. CARDINAL POINTS

These focusing fields have a definite axial extension, even in the case of apertures, and thus correspond to "thick" optical lenses. Their imaging action can be represented by a set of cardinal points, just as in the latter, and the same optical formulae can be used. For weak lenses it proves to be adequate to use the simple form: $1/f = 1/u + 1/v$, where u is the object distance and v the image distance from a lens of focal length f. The typical arrangement of cardinal points in electron lenses is rather different from that in normal optics, however, the principal planes being crossed over. Figure 6 shows their order and illustrates their definition. The principal plane is located at the intersection of the direction of incident parallel rays and their emergent direction produced back. As shown, the principal plane P_2 of image space falls actually before the center of the electron lens, and lies in the object space. Since ray paths are reversible in electrostatic lenses, as in optics, a similar plane P_1 is defined by a pencil of rays imagined as coming from the image space. The focal planes naturally are located where point images are formed by such parallel beams, F_1 and F_2, arriving from either direction. The distances of these from P_1 and P_2 respectively give the focal lengths f_1 and f_2; electrostatic lenses are usually symmetrical and $f_1 = f_2$.

If the distances of object and image from their respective focal planes are now p and q, Newton's relation holds true as in optics:

$$p \cdot q = f_1 f_2 \qquad (4)$$

This is the basic optical formula for calculating image positions in electron lenses for any given object position. Since the lens is not now a solid medium, it follows that the object may be situated actually within the lens,

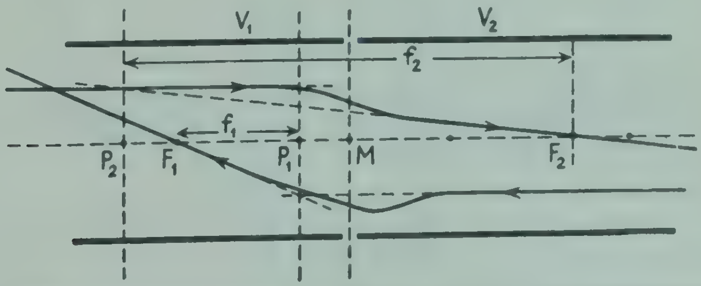


FIG. 6. Electron trajectories and cardinal points in a two-cylinder electrostatic lens ($V_2 > V_1$).

but it can be shown that Eq. 4 still holds. In a normal objective lens, the specimen may be in or very close to the center of the lens.

From experiments with electron lenses, and to a close approximation by calculation, the focal length can be found for any geometrical arrangement of electrodes and its variation with applied voltage followed. Such information will be found in the standard textbooks of electron optics (Cosslett, 1946; Glaser, 1952; Zworykin *et al.*, 1945). The important point to notice is that the focal length can be varied over a wide range simply by changing the voltages applied. In a strong unipotential lens the focal length will be roughly equal to the electrode separation (a few millimeters). Danger of electrical breakdown prevents smaller focal lengths being obtained, and makes the use of electrostatic lenses less reliable than that of magnetic lenses.

3. MAGNETIC LENSES

The action of magnetic fields on electrons is more complicated than that of electric fields. The difference may be pictured thus: in an electrostatic field electrons have a tendency to follow the lines of force, whereas they tend to spiral around magnetic lines of force. The focusing effect of a magnet is roughly speaking that of a potential trough between the north and south

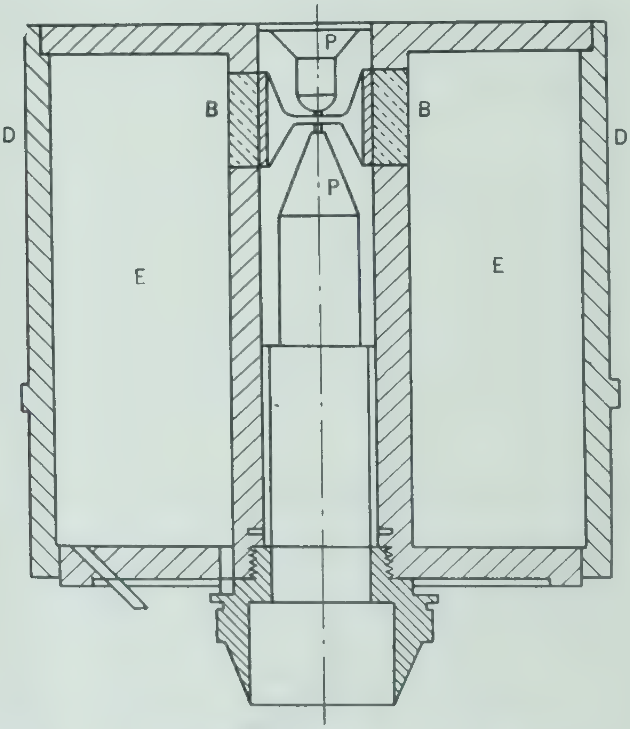


FIG. 7. Magnetic objective lens (axial section).

poles—if these are hollow and an electron beam enters through one of them (Fig. 7). But the spiralling influence causes the electrons to move around the axis at the same time as they are urged towards it. The result is that the image is rotated by an arbitrary angle with respect to the object, depending on the field strength and the electron speed, compared with the simple inversion of image that occurs in an optical or electrostatic lens. It is also found that a magnetic lens has only "troughs" and no potential "ridges," so that both halves of the lens field have positive focusing action. Hence magnetic lenses are always positive (= convex); they also all have aberrations of positive sign, so that correction cannot be carried out by matching positive and negative components, as in glass optics.

The typical construction of a magnetic lens is shown in Fig. 7. Polarity is produced in an iron casing D by current flowing in a slab winding E of many thousand turns of copper wire contained within it; permanent magnets can also be used. The magnetic field is concentrated into a small region on the axis by fitting conical polepieces PP of soft iron into the upper and lower parts of the axial casing, separated by a brass ring B. The focal length in a strong lens is roughly equal to the bore or separation of the polepieces, which are usually about the same; the minimum value is rather less than 1 mm (Liebmann, 1952). The focal strength depends on the geometry of the polepieces and on the current I and number of turns N in the winding (ampturns). In a given lens the relation is in first approximation:

$$f = Kv/I^2 \tag{5}$$

where K is a constant and v is the voltage by which the electron beam is accelerated. In the strong lenses of electron microscopy K does not remain constant as I increases. The typical variation of f with I is shown in Fig. 8—the focal length at first falls rapidly and then approaches a minimum value. Practical lenses naturally operate at the highest currents allowable without special cooling, so as to approach their minimum f; one or two models in fact employ water cooling. With too high an excitation, however, saturation of the iron may occur and the focal length increase again.

The disposition of principal planes in magnetic lenses is the same as in Fig. 7. The normal optical constructions for ray paths may be used and the thick lens formula again holds good (Eq. 4); but it must be noted that the effective focal length of a strong lens is different when used as projector from what it is when used as objective. The distinction is illustrated in Fig. 9, where a ray parallel to the axis is bent so strongly as to cross it within the lens, at F_o. A highly magnified image will be so far away that the emergent rays are effectively parallel, and so Fig. 9 with arrows reversed shows the behavior of the lens as objective: an object placed at F_o will be the origin of a beam passing out to the right. The principal plane for such an objective

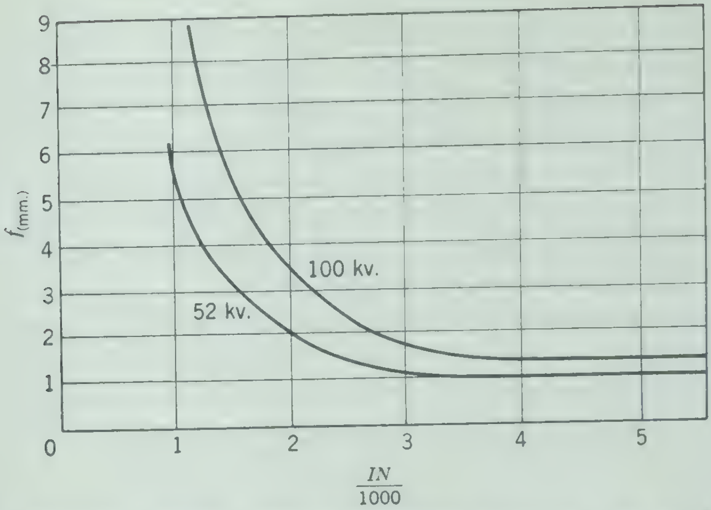


FIG. 8. Variation of focal length of magnetic objective lens with excitation (amp-turns, IN), for two beam voltages (52 kv and 100 kv).

will be at H_o and the focal length is f_o. On the other hand, in the projector the beam enters almost parallel to the axis, as from the right in Fig. 9, will be bent to cross the axis at F_o, and will emerge at the left strongly divergent. The principal plane of the lens as projector H_p will be where this direction, produced back, cuts the parallel entrant beam; H_p is clearly further back than H_o, and the projector focal length f_p correspondingly greater.

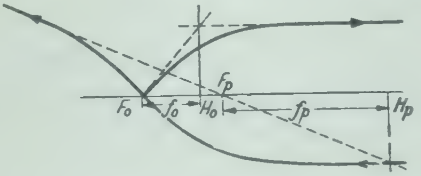


FIG. 9. Ray paths, focal points and principal planes for objective and projector lens conditions.

Thus a projector lens so strong that the beam crosses the axis within it will actually have a longer effective f than a weaker lens. If the lens is so strong that the beam crosses in the center of the lens, it will emerge parallel to the axis, being bent outwards in the second half of the lens by exactly the same amount as inwards in the first half; the focal length would then be infinitely great. Hence Fig. 8 shows only the variation of f with I for an objective lens, where the object is always very close to the focal plane. A projector lens shows a similar diminution of f with I at first, but at high excitation it passes through a minimum and rises again. As the projector is used to vary magnification, this means that for high currents a diminution of the final image size may actually be observed.

4. ABERRATIONS

Magnetic and electrostatic lenses both suffer from the same variety of aberrations as do optical lenses; the magnetic type has three additional errors due to image rotation. The most serious effects in practice are spherical and chromatic aberrations and astigmatism, all of which are severe by optical standards and rather worse in electrostatic than in magnetic lenses.

a. Spherical Aberration

The cause of this image-marring defect is that the outer zones of the lens are relatively too strong compared with those near the axis, so that the image formed by the former is closer to the lens than that formed by the latter zones. A sharp image can only be obtained by stopping down the lens until a small axial zone alone is used for focusing, with corresponding reduction in intensity of the imaging beam. It is found theoretically that this aberration is inherent in all electron lenses of the simple type now used, and could only be eliminated in a much more complicated system.

The practical effect is that what should be a point image is enlarged into a "disc of confusion" of radius R_s given by

$$R_s = C_s \cdot \alpha^3 \tag{6}$$

where C_s is a factor depending on the lens design and approximately equal to the focal length; α is the angular semi-aperture of the lens (cf. Fig. 1). Hence reducing the aperture rapidly reduces spherical aberration, but as this is done the diffraction effect increases, as indicated by Eq. 2. In practice a compromise in the size of stop is made such that R_s and R_D are made equal by the value of α chosen. In a strong objective α will be about 5×10^{-3} and the diameter of the stop 60 to 100 microns. This is the reason for inserting apertures in electron lenses, with the consequent difficulties of centration and regular cleaning. As indicated in Section III, it is possible to obtain good resolution without an aperture provided that attention is paid to obtaining illumination conditions that in effect provide a beam of proper angular aperture, but it is easier to use a stop.

b. Astigmatism

Astigmatism arises if the focusing action of the lens is stronger in one direction across the axis than in that at right angles; it is equivalent to the bore being elliptical instead of circular in section. The inherent astigmatism of an electron lens is negligible compared with the spherical error, but a much larger astigmatism is unavoidably introduced by technical limitations in making the lens. In a magnetic lens this may be due to two causes: the bore may not be truly cylindrical, or the magnetic material may not be absolutely homogeneous, owing to blow holes, inclusions or other faults. The resulting asymmetry of the field gives an astigmatic image: a point object gives two sharp line images, at right angles to each other and to the axis, and separated from each other along the axis. The sharpest representation of the object will be a circular disc of confusion half way between the line foci. The visible effect in the electron microscope will be that the image seems to streak out first in one direction and then in that at right angles as the objective is varied through focus. Even when the astigmatism is insufficient to cause directly visible elongation, it may be enough to be the limiting factor on resolution, rather than spherical aberration.

Fortunately this astigmatism can be corrected by introducing fields that are asymmetrical in the opposite sense to that of the lens. This is done by inserting radial iron screws and adjusting their position until correction is obtained, as a result of careful observation of diffraction fringes in the image of a known object. The full procedure is given by Hillier and Ramberg (1947), but some microscope makers now supply lenses already corrected. If astigmatic line foci suddenly appear from a lens that has previously been free of them, it is an indication that an asymmetric field has arisen somewhere along the beam path, most probably by electrostatic charging of an aperture which has become contaminated (cf. Section VIII).

c. *Chromatic Aberration*

From Eq. 5 it is seen that the focal length of the lens depends on the beam voltage as well as on the strength of the magnetic field, as given by the current. Any variation in either v or I will change f and thus confuse the image; this is what is meant by chromatic aberration. Electrons of different voltage have different speed and so will be brought to different focal points; the faster the electron, the "stiffer" the beam and the longer f. The beam voltage must be held constant to such a degree that the disc of confusion arising from the spread of velocities is smaller than that due to spherical aberration and diffraction combined. In practice this means a degree of stabilization better than 1 part in 80,000, for a resolution of 10 Å. Hence the need for complicated electronic stabilizing circuits—and for ensuring periodically that they are functioning properly. Similarly the lens current supplies must be highly stabilized, so that the focusing fields do not vary in strength. An accumulator bank is stable enough for resolutions down to 40 Å, but for better performance electronic stabilizing gear is essential.

A small loss of stabilization is difficult to detect from the appearance of the image, and can only be found by direct check on the stability of the circuits with a cathode ray oscilloscope. A large chromatic error will cause blurring of the image, which will increase with distance from the axis. It is in practice more important to guard against voltage than current troubles, so that the alignment of the microscope is now usually carried out whilst applying variations of voltage; an equivalent test is to reverse the objective current (cf. Section VIII).

V. The Illuminating System

1. The Electron Gun

In practically all electron microscopes the illumination is provided by a white hot tungsten filament *F* (Fig. 10), surrounded by a negatively biased shield *S* through an aperture in which the beam is drawn off to a positive anode cap *A*. The exact shape of the electrodes varies, but we need only consider here the essential features of the electron gun. The detailed effect of the electrode parameters and applied voltages on the properties of the beam is given fully by Haine and Einstein (1952).

In an electron gun of given design, the filament temperature determines the total electron emission whereas the bias voltage on the shield controls the angular spread of the issuing beam and hence its brightness. The condenser lens concentrates this beam on to the specimen, giving an additional control of illumination. The shape and position of the filament also affects the brightness, so that great care has to be taken when changing it. The gun controls proper will be first discussed, and then the optimum use of the condenser.

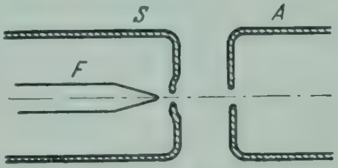


FIG. 10. Electron gun for electron microscope (axial section).

a. Filament Position

The gun of a given electron microscope is designed for a given position of the filament; it must be on the axis and at the correct distance within the shield aperture. The latter distance ("height") is not highly critical in biased guns, and a simple jig allows it to be checked before inserting the filament in its holder. But the axial position must be more exact than can be pre-set in this way, and also it may vary when the filament is heated for the first time. An off-axis displacement of 0.001 inch will tilt the beam so much that it may miss the condenser aperture altogether.

When a filament has to be inserted, it should first be adjusted by eye in a jig or dummy shield, then heated in a flame or by passing low current, and re-adjusted. The makers give instructions as to the correct height. After mounting in the shield, it must be re-examined under a low power lens and set as closely as possible on the axis. Any small residual off-axis error can be corrected by the tilt mechanism of the illuminating system, as described in the alignment procedure (Section VIII).

b. Filament Temperature

The current through the filament is controlled by a resistance and is usually indicated on a meter. Increase in current raises the filament temperature and thus the electron emission. The electron illumination increases in brightness up to a limiting maximum if the maximum voltages applied to the anode, the shield and across the filament itself are fixed. In fact the "ceiling" is reached quite rapidly, since the electron microscope gun is usually self-biased: the beam current flows through a resistor inserted between filament and shield, so that the potential drop across it provides the biasing voltage. The larger the current drawn from the filament, the larger this drop and the higher the bias, so that as the filament temperature is raised a condition is soon reached where the bias throttles the emission. Bias-saturation takes the place of the space-charge saturation of an ordinary triode.

Hence, for a given value of bias resistor, a maximum is fixed to the beam current that can be obtained. Illumination is controlled during microscopy by varying the filament current; in order to spare the specimen, viewing should be begun with minimum current, which is then increased up to the value needed for observation at the electronic magnification chosen. If the bias is independently variable, its control should also be adjusted for minimum illumination initially. Working with no more than just sufficient brightness for viewing prolongs the life of the filament as well as minimises damage to the specimen.

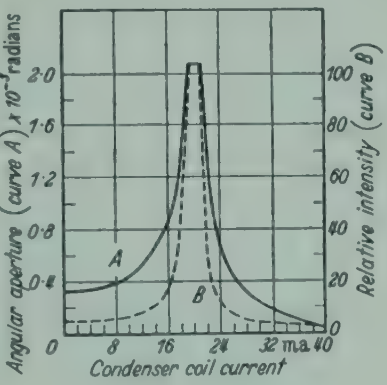
c. *Bias Voltage*

As explained, the bias voltage is usually supplied by the potential drop down a fixed resistor, the value of which is so calculated as to provide an adequate range of illumination when the filament current is varied. In some models a variable bias is provided in the form of a set of resistors which can be switched into circuit in turn. This has the advantage that any small error in filament height can be offset by varying the bias. Also the filament current can now be kept fixed at a moderate value, consistent with long life, and the intensity of illumination varied by selecting different bias resistors. The effect of changing bias is to concentrate the beam emerging from the gun into a wider or narrower solid angle. If the bias is radically changed, it may be found that the filament current also needs slight adjustment. On the other hand, if the gun is not self-biased, but the necessary voltage is supplied from a separate source, the beam will not be automatically throttled as the filament current is raised, and greater care is necessary to avoid specimen damage.

2. CONDENSER LENS

The condenser lens is located approximately mid-way between filament and specimen. It will give brightest illumination, for given gun conditions, when its power is such as to focus an image of the filament on to the specimen ("critical illumination"). The illuminated area on the latter will then be equal to the effective size of the filament, about 50 microns in diameter. This condition is to be avoided if at all possible, since it is liable to give too great heating of the object and also the illuminated field may be uneven in brightness, owing to variations in emission across the filament ("structure"). As the power of the condenser is varied, by changing the current through it, the image of the filament is formed either above or below the object plane and the illumination there is less intense than at critical focus, since the beam accepted by the condenser is now spread over a larger area of specimen. The corresponding variation in the angular aperture of the illumination at the object is given in Fig. 11. It is constant at a maximum value α_x over a small range of condenser current about critical focus, and falls off sharply at higher or lower current. The beam intensity at the specimen is proportional to the solid angle, and hence to α^2; thus it is even more steeply peaked than the variation in α.

FIG. 11. Variation in (A) angular aperture of illumination and (B) intensity, with condenser lens current, i (mamp).



In normal operation of an electron microscope the condenser current should be above or below the peak value, to spare the specimen. It is brought towards the critical focus to provide strong illumination for sharply focusing the image, but the condenser should be "backed off" again for photography. This procedure not only reduces the total load on the specimen, but also increases the sharpness of focus of the finally recorded image, since the depth of focus increases as α is decreased and the aberrations are less pronounced (cf. Eq. 6.).

VI. The Imaging System

1. SPECIMEN CHAMBER

The specimen to be examined is mounted on a supporting metal gauze (Section IX), which is placed in a metal cap or similar holder and inserted firmly into the stage plate of the electron microscope, movable in two mutually perpendicular directions by push rods for searching the specimen. The early instruments contained an air lock by means of which the object holder could be transferred to the evacuated interior without admitting air; a separate pumping line was provided for evacuating the lock before opening it up to the rest of the apparatus. Most models now have either a pencil type holder that pushes in through a rubber seal with hardly any admission of air, or else have such fast pumps that the whole column can be let down to atmospheric pressure when the object is changed. In the latter case the prescribed switching routine of the vacuum taps must be followed.

The object holder must always fit firmly in its seating, so as to minimize vibration and to aid thermal contact. The stage plate is moved by controls operated from outside the machine, and should travel smoothly and without appreciable back lash even when observed at 10,000 magnifications. Stops are fitted to ensure that the specimen grid cannot be carried right out of the beam, whilst allowing practically all of it to be viewed. The controls must never be forced in an effort to see a remote corner of the grid, as this may deteriorate the vacuum seals or even damage the stage plate. It is advisable to remove and polish the latter and the surfaces on which it moves at least once a year, or more often if there is any suspicion of sticking.

Most holders now have an attachment for stereophotography, by which the specimen grid is tilted through a small angle between two successive exposures. This is readily operated if so designed that the image can be observed during the tilting process, so that the wanted feature may be kept in the field of view by moving the stage controls. But the two-position type of stereo holder makes necessary a subsequent search to find the wanted field again after tilting; it is best to make a rough drawing of it before doing so.

Holders must always be kept clean and polished, to avoid dirt reaching the specimen and to ensure good thermal and electrical contact between grid and holder. In a run of work where the magnification should be the same, it is essential to employ the same holder right through since a slight difference in axial dimensions will displace the object by that much from the assumed object plane. In the usual type of objective lens, a change of $\frac{1}{1000}$ inch in object distance will make a difference of $\frac{1}{2}\%$ in magnification.

2. Focusing and Magnification Change

When the object holder has been placed in position, the image is focused by means of the objective current controls, which usually comprise a coarse and fine motion; sometimes an ultra-fine control is also provided. The magnification is varied, in a two-stage instrument, by changing the projector current; doubling of its object distance ($\fallingdotseq$ focal length) will give a similar change in final magnification, as the projector-image distance is fixed (cf. Fig. 3). The depth of focus of the first image is usually so great that no corresponding change of the objective current is needed, and the variation in the distance OI_1, is relatively so small that the magnification is controlled only by the projector. Hence some microscopes have the projector current meter directly calibrated for magnification. Any such reading is not likely to be accurate to better than 10%, however, unless regularly checked (cf. Section VII).

In practice it is usual to start at low magnification in order to survey a wide field and select features of interest in the specimen for greater magnification. As this is done, the intensity of illumination must be increased *pari passu* to keep the image bright enough for ready observation. When a field has been chosen for photographing, it is focused as sharply as possible by using the fine objective control. As already mentioned, it is advantageous to use a low-power viewing telescope for the purpose, and on many instruments they are now standard fitments. A properly designed optical system presents a magnified image without loss of brightness, and so permits microscopy at a low electronic magnification and small object loading, whilst the image is observed at the magnification to which the photograph will be finally enlarged. But there is no point in using an optical magnification greater than makes visible the graininess of the viewing screen, and a ×10 telescope is adequate with normal screens. A monocular will suffice, but a binocular viewer is to be recommended. When high performance is sought, the telescope enables diffraction fringes to be viewed directly, and, when used with high electronic magnification, helps in the detection of minute vibrations or drifting of the image.

3. Depth of Focus

When the image is well focused it is good practice to change the condenser control so as to diminish the illumination appreciably for photography. The angular aperture is thus reduced (Fig. 11), the depth of focus increased, and the resolution improved. Alternatively some microscopes have a means of artifically *increasing* the value of α during focusing by electrically displacing the beam. In either case experience will show whether the objective setting is changed in the process, demanding a "blind" re-adjustment of the fine focus control before exposure.

The meaning of depth of focus is illustrated in Fig. 12. O is a point in the object through which an illuminating beam of aperture 2α passes. In the image the resolution may not be sufficient to define O itself, the least observable distance d being greater owing either to lack of true resolving power in the lens or to graininess in the screen or poor visual acuity. Dividing d by the magnification gives the equivalent detection limit in object space, d/M. Figure 12 shows that the objective may be focused anywhere between

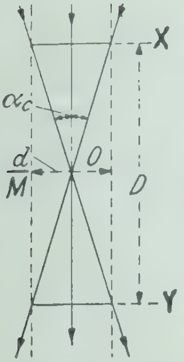


FIG. 12. Depth of field within which an object O is imaged with a given degree of confusion.

the planes X and Y, which give an intercept d/M across the illuminating beam, without visible change in the image. The axial separation XY is properly defined as the "depth of field" corresponding to the practical resolution d/M, but is usually called the depth of focus. We have: $d/M = \alpha \cdot XY$. If now the value of α is reduced by varying the condenser, the depth of field XY increases in proportion and there is a greater chance of getting the recorded image adequately sharp. By this method, by use of a viewing telescope, and by bracketing the image by varying the fine focus and setting it at the mean position it should be possible to get results sharp enough for all normal purposes. For very high resolution work it may be necessary to take a through-focal series of photographs with blind settings of the fine focus control, progressively increasing in value (Section VIII).

The term depth of focus properly refers to the image space, where a similar effect occurs but in a much greater degree. Here the transverse intercept on the beam is greater than in object space by the factor M and the beam angle is smaller by the same factor, since from geometrical optics $I/O = M = \alpha_o/\alpha_i$. Hence the depth of focus in the image is M^2 greater than in the object: with M of the order of 10,000 this gives a factor of 10^8. Consequently the image is in focus practically from the end of the projector lens right away to infinity. Therefore the position of the photographic plate is not at all critical. The recording plane can be well below the viewing screen, which can thus be used as a shutter; or it can be much nearer to the projector, as in the Philips microscope, with smaller picture and shorter exposure.

The advantage of great focal depth follows from the minute apertures imposed on electron microscopy by the aberrations of its lenses. In optical microscopy the large numerical apertures make the depth of focus extremely small, even at the poorer resolution attainable, so that sections have to be examined plane by plane. In the electron microscope, where the specimen has to be thin in any case, the whole of it is in focus at once and discrimination in depth is only possible by stereo-operation.

4. THREE-STAGE MICROSCOPES

Since Marton (1945) first constructed an instrument with three stages of magnification both the Philips and Metropolitan-Vickers electron microscopes have used this arrangement. By having two independent projector lenses, the magnification can be varied continuously over a wide range and electron diffraction can be practised. As it is probable that most makers will go over to this system in future, it is in place to describe it here. Figure 13 shows typical modes of operation of the three-stage microscope, according to the relative strengths of the objective and first projector (inter-

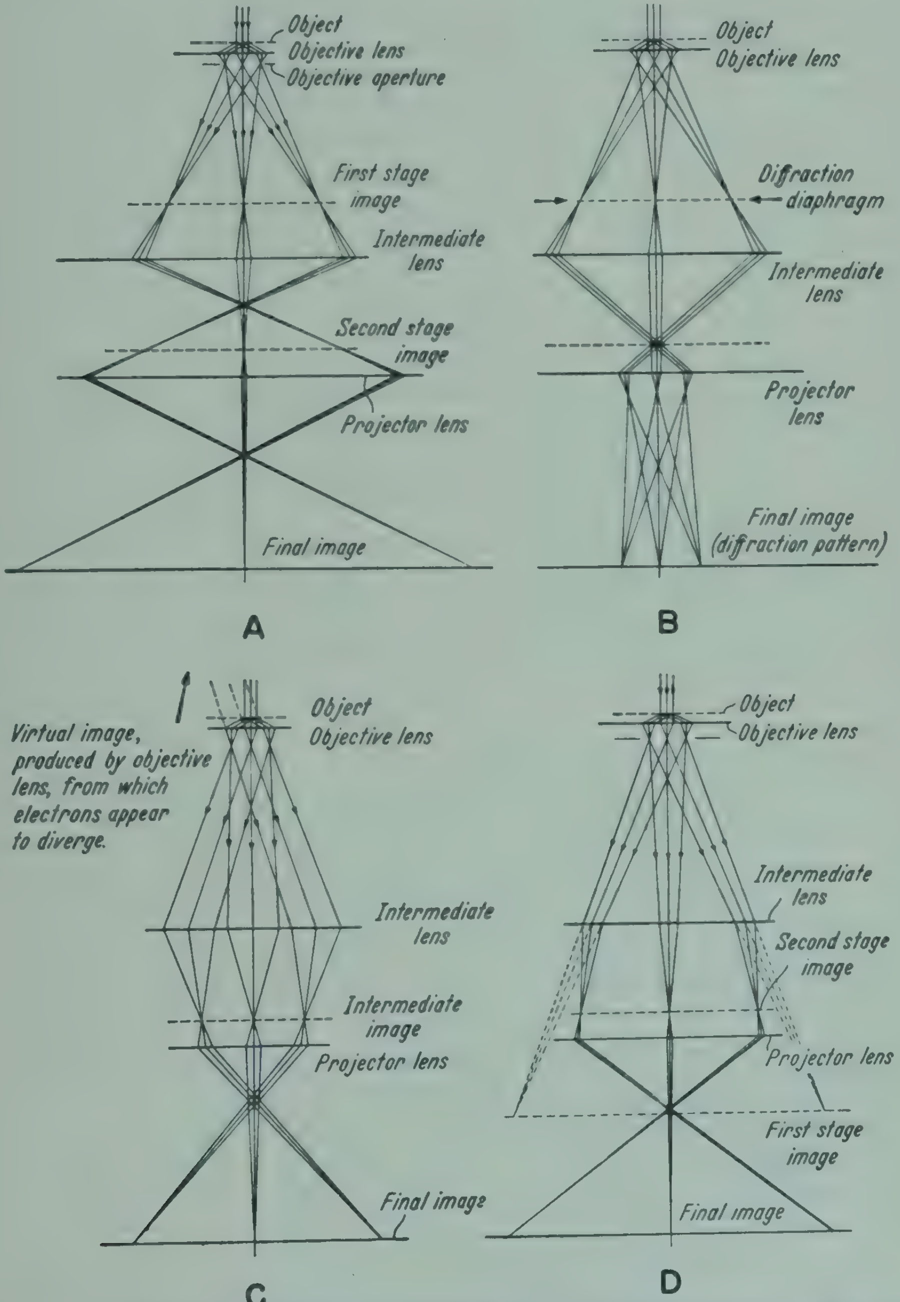

Object
Objective lens
Objective aperture
First stage image
Intermediate lens
Second stage image
Projector lens
Final image
A
Object
Objective lens
Diffraction diaphragm
Intermediate lens
Projector lens
Final image (diffraction pattern)
B
Virtual image, produced by objective lens, from which electrons appear to diverge.
Object
Objective lens
Intermediate lens
Intermediate image
Projector lens
Final image
C
Object
Objective lens
Intermediate lens
Second stage image
Projector lens
First stage image
Final image
D

FIG. 13. Three-stage electron microscope: (A) high magnification, (B) diffraction, (C) first low magnification range, (D) second low magnification range.

mediate lens); the second projector is run at maximum power throughout, to minimize distortion and keep the field of view filled. For high magnification the objective is operated at high power and the intermediate lens forms an intermediate image (Fig. 13A), so that the total magnification is given by the product of three stages, of the order of 20×, 5× and 100× respectively. For diffraction operation the intermediate lens is reduced in power so as to throw a practically parallel beam into the projector; this is equivalent to forming an image of the electron source on the final screen, so that the diffraction pattern arising from it is also seen (Fig. 13B). For low magnification, the objective power is reduced to give either a first image below the intermediate lens, which the latter then reduces (Fig. 13D), or a virtual first image which is similarly reduced by the intermediate lens (Fig. 13C). Specimen figures are given by Challice (1950) which show how the magnification range can be continuously covered. In practice the image below about 500× is distorted so severely that it is useless; but it is possible to cover a range from about 500× to 60,000× simply by moving control knobs. It is also a great convenience to be able to get diffraction patterns from an object first surveyed at high magnification. A small aperture below the specimen allows diffraction to be obtained from an area some few microns only in diameter, so that the components of a mixture can be identified. The three-stage instrument can also be used for two-stage operation, of course, by switching out the intermediate lens. Its care and maintenance is essentially the same as that of a two-stage instrument.

VII. Recording the Image

1. Photographic Materials for Electrons

The image focused on the viewing screen is photographed on film or plate either by lifting the screen or by turning the camera into the beam (Philips model). With the emulsions generally used the exposure time is of the order of a second, so that no high speed shutter mechanism is needed and the screen itself usually serves as shutter. Most cameras provide for a small number of successive exposures without reloading, some provide only a single plate, and a very few use a length of film on which a large number of exposures may be made. The loading of the camera is usually very simple, a cassette being first charged with plate or film in the dark and then transferred to the microscope in daylight. The camera chamber is isolated from the rest of the apparatus by an air lock, so that re-loading can be effected without admitting air to the rest of the column, and often without interrupting microscopy. The large water content of most emulsions makes it necessary to pump for several minutes before the camera chamber can be opened up to the column. This delay can be appreciably shortened if the loaded cassette is pre-pumped under a bell jar at a rough vacuum for some time before transfer to the microscope.

The photographic material most used for electron photography is of the lantern type. Kodak B10 or B20 and Ilford Special Lantern plates have the right speed and contrast characteristics. For specimens lacking in contrast, or when no aperture is used in the microscope, it may be preferable to use an emulsion of the type used for document copying, such as the Photomechanical plate; this has great contrast but is about five times slower than the lantern plate. In film, the Kodak Microfile Regular and Ilford 5B11 have good photographic properties and pump out relatively rapidly; Kodaline has also given good results. When ordering, it is desirable to specify non-panchromatic material since otherwise dark-room work is unnecessarily complicated; the ordinary orthochromatic emulsions are perfectly satisfactory for electrons. Some makers offer for electron microscopy material which is normally panchromatic but from which the sensitizer has been omitted.

Normal development and fixing is employed, and special fine-grain developers can be used when the need arises, as for light images. In general the graininess to electrons seems to be much the same as to light, and subsequent enlargement of from seven to ten times is practicable with lantern plates. This corresponds to the magnification usually employed in the telescopes used for focusing the image. There is no point in using finer grained material than the accuracy of focusing warrants, unless "blind" photography by a through-focal series is adopted (Section VIII). The few investigations so far carried out on the sensitivity of emulsions to electrons show that the range of speed is much less than for light. If a finer grain plate than a lantern type is to be used, therefore, it must be remembered that it is likely to be relatively faster than when used for light. The recent work of Möllenstedt (1951) on high resolution plates indicates that the line resolution is not quite as good for electrons as optically.

2. Magnification Calibration

A given electron microscope will be provided with a magnification calibration chart, usually in terms of the projector current but sometimes referred to the size of the image of an aperture or fine wire in the projector. For all normal purposes where an accuracy of no better than 10% is required this chart will be adequate. The actual magnification will vary according to the position of the specimen, the voltage employed and possibly magnetic changes in the pole pieces. When greater accuracy is demanded, as when investigating particle size distribution, a special magnification calibration must be made in the range of working conditions to be employed. Unless care has been taken to make all object holders of exactly the same dimensions (to better than 0.1 mm), the same holder must be used throughout and the specimen grid always inserted in it in exactly the same way.

Various accurate methods of measuring magnification have been devised, using diffraction grating replicas or quartz fibers of a convenient size that can be checked by optical means (cf. Cosslett, 1951, pp. 135-142). For most purposes, however, it is simplest and sufficiently accurate to use standard reference objects, of known size, the two most suitable being polystyrene latex spheres and tobacco mosaic virus rods. A particular batch (580G) of Dow latex was found to be of extraordinarily high uniformity in size and thus ideal for magnification measurement. It can be added in small amount to many actual specimen preparations, so that reference particles appear on the electron micrographs. Or it can be used in a separate calibration series of photographs taken at various settings of the projector controls. Small samples of the latex may be obtained either direct from Dr. C. H. Gerould, Dow Chemical Co., Midland, Michigan, U. S. A., or through one of the national electron microscope organizations. There has been some confusion as to its exact diameter, the cause now having been traced to the presence of some other substance in the suspension, which adds a coating to the particles during drying on the specimen mount, the thickness depending on the dilution employed. If it is deposited direct from the concentrate as supplied (40 %), the diameter is found to be 2600 ± 25 Å, as a mean of several measurements by electron microscopy (Gerould, 1950), with which low angle x-ray methods agree well (Danielson *et al.*, 1952). But if it is deposited from high dilution, there is very little extraneous material per particle and the diameter is found to be 2420 ± 50Å (Cosslett, 1949; Ellis, 1952). It is therefore desirable to adopt a standard procedure corresponding to one or other of these results.

For high magnification work the latex particles may be found too large; at 20,000× one of them will give an image 5 mm in diameter. The most suitable material is then one of the plant viruses, of which tobacco mosaic virus has the most uniform size. Its rods vary in length, but in width are 150 Å when dried down on the specimen support and very lightly shadowed; without shadowing the contrast is too poor for accurate measurement. Care must be exercised to avoid adding appreciably to the width by heavy shadowing, as the amount of metal conventionally used in most laboratories will add tens of Angstrom units to the width of a projection such as a virus rod. A more accurate reference distance of this order of size would be the lattice of the crystals formed by virus particles and many proteins, paraffins, and other macromolecules. The technique of preparing them is not yet well enough developed for anyone but an expert in the field to employ, but recent advances hold out the promise of this very exact measure (which can be checked by x-rays) becoming a standard for electron microscopy (Wyckoff, 1949; Dawson and Riley, 1951). The smallest lattice clearly resolved and measured by the electron microscope is that of Edestin, with a repeat distance on the 111 faces of 70 Å (Hall, 1950).

3. Resolution Estimation and Limits of Detection

Nothing is so difficult in electron microscopy as getting a reliable measure of resolution. It is natural for an investigator to be over optimistic about his results, but even an impartial group of observers will give quite different estimates of the resolution in a particular micrograph. The difficulty is that the proper definition of resolution (Section I, 3) can only be applied to two closely neighboring object points of radius equal to the linear resolving power. In most preparations it will be most unlikely that particles of the right size occur, still less that two will be in contact edge to edge. By using a finely divided colloidal suspension (gold or silver) the resolving power of an electron microscope can be tested, since particles of all sizes down to about 25 Å can be obtained. But it is not practicable to add such material to all preparations as a regular method, and other measures of resolution have to be devised for general application. The sharpness of angle where two rods or edges overlap can be employed, or the width of the photometer trace across the image of an opaque edge, the latter giving a direct comparison with the true resolution as previously defined. The applicability of these and other measures is discussed by Cosslett (1951, pp. 142–148).

The most generally useful estimate of resolution, however, is from the size of the smallest particle or other feature that is definitely visible in the image. This limit of detection is best determined by making successively greater enlargements from the negative, until the size is reached at which the image is no longer judged to be sharp when viewed at the distance of distinct vision. Taking the resolving power of the eye as 0.2 mm, the limit of detection is then $0.2/M$ mm, where M is the maximum useful magnification. For instance, if the image is judged to be sharp at 40,000× but not at 50,000×, the resolution is 50 Å. This method is essentially the same as that of "quality estimation" proposed by Hillier and Baker (1945). It gives a figure considerably smaller than the resolving limit as properly defined, since the eye can detect an isolated object much more readily than it can separate it from an overlapping neighbor. For opaque disc-like objects the detection limit should be multiplied by a factor of 3 to get an approximate estimate of the true resolving power. For line objects the factor is of the order of 10. There is thus an appreciable difference between the limits of resolution and detection, which often leads to exaggerated claims of performance.

VIII. Requirements for High Performance

In order to get the very best results from an electron microscope attention must be paid to a number of disturbing influences which may affect the image. Special care needs to be taken in focusing and photographing, and also in the exact alinement of the lenses. These points of refinement will be discussed in turn. (See also Haine and Mulvey, 1954 and Leisegang, 1954).

1. MECHANICAL STABILITY

The need for mechanical stability is obvious if a high resolution image is to be recorded. Vibration of the microscope as a whole is of no account provided that specimen and photographic plate are rigidly connected, so that one does not move relative to the other during exposure. Motion of the specimen is most deleterious, since it is magnified highly in the image. To minimize it, the specimen grid must be firmly held in its holder and the holder firmly fixed in the specimen stage of the microscope. The latter should be designed to ensure this, but small particles of dust or of damaged specimen may hinder proper fit. All the surfaces involved must be kept regularly cleaned and polished.

However, the stage has to be free to move under external control and it it is very difficult to combine this requirement with an absence of play. Even if the stage motion and bearing surfaces are kept in good order, there may remain some slight sticking friction. It is good practice to tap the stage controls lightly before making an exposure; if the image is watched during the process it will soon be found if this is a desirable routine procedure.

In spite of all care there is bound to remain some degree of play of the specimen with respect to the plate, if only in the order of tens of Ångstroms, when the microscope is subjected to external vibration. This appears to be one of the major practical limitations on resolution, rather than the theoretical aberrations of the lenses (Haine and Mulvey, 1950; 1951). For high performance every source of vibration must be kept away from the microscope. The rotary vacuum pump should be as far away as is consistent with good pumping speed; the best solution is to install a vacuum reservoir between it and the diffusion pump, and switch it off during exposure. The diffusion pump heater supply should be checked to ensure that the fluid is not boiling too rapidly, giving undue "bumping." Any other machinery or apparatus causing vibration should be kept remote from the microscope, which itself should preferably be situated on a firm foundation, e.g., in a basement rather than on a higher floor. Finally the shutter must be operated as smoothly as possible during exposure, to avoid jarring the column. The exposure should not be less than one second, otherwise jerky action is unavoidable; it should not be greater than two or three seconds, otherwise the probability of external disturbance becomes too high. Photographic material of suitable speed must be chosen to give an exposure of one or two seconds.

2. ELECTRICAL STABILITY

The high voltage supply and the current in the lenses must be adequately stabilized, both as regards ripple and longer term fluctuations.

Stability must be of the order of 1 part in 50,000 for a resolution of 20 Å. This requires the services of an electronic specialist to test the circuits under operating conditions; it is not sufficient to test them under no-load conditions. Testing the lens supplies is not difficult, but for the high voltage a condenser potentiometer must be set up to tap off a suitable fraction of the total voltage. The tuning up of the circuits to this degree of performance is in fact best done by a servicing engineer well familiar with the circuits employed.

Apart from variation in the microscope supplies, the image may be blurred by external alternating fields and distorted by constant fields. Trouble most often arises from stray magnetic fields from transformers, heavy current lines, etc. The microscope column is fitted with protective mumetal shielding, but this may not be adequate against strong stray fields when high performance is demanded. The magnitude of such effects can be found by exploring the space around the microscope with a coil of many turns (50,000) connected to a sensitive cathode ray tube. The influence of the field on the image can then be estimated by applying a known field in the same region and observing the amount of blurring. Haine and Mulvey (1950; 1951) have described a method of testing for minute residual effects of mechanical and electrical vibration by observing diffraction edges in the image.

3. Thermal and Electrostatic Stability

In addition to disturbance from external electric and magnetic fields, parts of the interior may become charged and so cause electrostatic deflection of the image. This trouble cannot arise if all surfaces struck by the beam are conducting, but it is found in practice that a non-conducting carbonaceous deposit gradually forms on any such surface. It appears to come from residual organic vapors in the column, and can only be avoided by raising a surface above 200°C (Ennos, 1953). Thus the specimen and supporting grid, one or more apertures and possibly the specimen holder are likely to acquire a deposit, most rapidly where the beam intensity is greatest, i.e., on the objective aperture. The condenser aperture usually receives a load sufficient to heat it above the deposition limit.

The visible symptom in the microscope image is flicking of the whole picture, usually repeating at intervals of a second or so, as charge accumulates and leaks away. Before this stage is reached, however, the aperture may already have an asymmetric coating which causes astigmatism; this is only likely to be noticed in high resolution operation, where diffraction fringes can be studied. The remedy is to remove the aperture and clean it according to makers' instructions. If it is of platinum it can be flamed; if of copper, mild attack with formic acid may be adequate. With skill, a ground-down needle may be used to ream out the hole, which is liable to be slightly enlarged in the process; if optical examination shows it to be appreciably non-circular, it should be rejected. If cleaning the aperture has no effect, the object holder should be treated and finally the polepiece itself. If the specimen contains large particles of non-metallic nature, it may well be that the trouble arises in the area irradiated. This can easily be tested by repeating observations with a blank specimen grid. Apertures further down the column need attention less often, as the current density is much lower there; however, trouble occasionally arises from small hairs or parts of specimens falling into the projector aperture. It is necessary for all parts that have been cleaned to be washed in ether or other very pure solvent before being replaced in the microscope.

Regular fluttering of the image is probably due to a contaminating deposit; an irregular effect is most likely due to trouble in the electron gun. It may be due to mechanical instability of the filament, or to deposits inside the shield. The latter may be rubbed off with fine emery paper, and the shield aperture similarly treated. From time to time the outside of the shield and the anode surface and aperture should also be cleaned. All such surfaces should be finished to a high polish, to minimize the danger of electrostatic breakdown.

Thermal instability of the specimen as well as electrostatic charging may result from the impact of the electron beam. An immediate break up of the supporting film may occur when the beam comes on, or there may be a gradual warming up of the whole specimen grid and holder, leading to a creep of the image. This proves to be a considerable hindrance to high resolution work, where the specimen should not move more than a few Ångstrom units per second. The most that can be done in a normal microscope is to ensure a close fit of all parts of the object holder to each other and to the support grid, after polishing all such contact surfaces. It is advisable to avoid woven grids, in which the separate strands of wire may move relative to each other under thermal load; plated, flat grids of copper are now generally available. For high resolution work, or if the specimen is very sensitive to electron bombardment, it may be best to use a massive disc as support, bored with a single aperture of 100μ or so.

4. Vacuum Conditions

Most commercial electron microscopes are fitted with a valving system such that the machine can only be operated if the vacuum has reached a certain limit, and if the vacuum worsens and causes the beam current to increase, a relay switches off the H.T. and so preserves the filament. It is good policy, however, to aim at getting the best possible vacuum. For one thing it prolongs filament life, for another it minimizes beam fluctuations.

Slight intermittent leaks of air, especially in the region of the gun, cause the illumination to shift and may spoil an exposure. More severe bursts may cut off the beam altogether, owing to overloading. Whenever such signs are observed, the gaskets and other vacuum seals should be gone over carefully to find the source of trouble. The instructions book should give details of how to go about leak-testing and how to replace gaskets or metal bellows. It is a sound rule to suspect that part of the apparatus that was last interfered with, e.g., when changing a filament. In the worst case, a systematic search must be made for a leak, starting at the top of the column, removing each unit in turn and sealing a glass or metal blank across the exposed opening. The rate of leak after pumping down may be observed each time on the built-in vacuum gauge; in this way the faulty component can at length be traced. It should be possible to get the system vacuum tight to the degree that, after shutting off the pumps, it does not rise above the working limit of vacuum for several hours; in some instruments an overnight "black out" can be attained.

5. ALIGNMENT

The permissible aperture of the electron microscope is so small (10^{-2} to 10^{-3}) that a high degree of precision is demanded in the alignment of the several lenses and the electron gun. The mechanical accuracy of construction can be made such as to ensure the correct parallel alignment of components in a two-stage microscope. Adjustments then have to be provided to bring the axis of each lens into line with that of the next, and to ensure that the gun delivers a beam correctly into the condenser. As mentioned, a tilt of the gun is needed since the filament tip cannot be accurately enough positioned in its aperture.

The problem sounds simple, so stated, and it might be thought that the whole column could be set up and clamped by the makers, even if the machining tolerances cannot be made fine enough to ensure exact fit on assembly. The complicating factor is that the magnetic axis of a lens may not coincide precisely with its geometric axis, and may vary with operating conditions and with time owing to hysteresis and saturation in the iron. It is usually necessary to re-align after changing the filament and advisable to check alignment after any radical change in operating conditions (voltage or lens power).

Two different principles may be used in aligning the column: that the image stays stationary when the lens power is varied, or when the beam voltage changes. Current centration has been most used in the past, but it is now appreciated that voltage alignment is more satisfactory for high resolution work, since slight variations are more likely to occur in voltage than in current. If the current and voltage axes coincided no choice would be needed, but this is hardly ever the case. Current alignment will first be described here for a typical two-stage instrument.

The criterion of centration is simple in a magnetic lens, since the image rotates when lens power or voltage changes. This makes centration in some ways easier than in electrostatic lenses, in other ways more difficult as the image may also be displaced sideways. This combination of effects hinders a straightforward deduction of remedial measures from the observed behavior of the image, as the objective current is varied about its normal value; it is preferable to isolate the rotational effect by reversing the current through the objective lens (Haine, 1947). An image point then jumps to a new spot on the screen, and the desired axis will lie half way between the two positions.

The first step in alignment is to remove the objective aperture and operate at low magnification, to get bright illumination on the screen. The condenser and gun traverse is used to keep this in view as the magnification is raised to a few thousand times; the image should expand and contract roughly symmetrically about the center of the screen as the condenser current is varied. Reverse the objective lens current and identify a point in the image which does not move on doing so. Move this point, which is on the axis, to the center of the field by shifting the projector (or objective, if the projector is fixed). The image should now expand about the center when the projector current is varied, and rotate about the same point when the objective current is reversed. It is desirable to leave the objective switch in the position such that the rotations produced by the objective and projector are in opposite senses.

If the image is now found to shift sideways as the objective current is slightly varied, without reversing, it is a sign that the beam from the condenser is not entering along the objective axis. Use the gun tilt controls to return a chosen image spot to the center of the screen, after it has moved away on reducing the objective current. After this has been repeated several times, the image should rotate about the center of the screen on varying the objective. Finally check that the brightest illumination is obtained in the center of the screen; if not, adjust the gun traverse controls until it is so. Now insert the objective aperture and align it according to the mechanism provided, using a low magnification image.

For voltage alignment the same procedure may be followed, but rapidly switching the H.T. on and off instead of reversing the objective. Hillier and Ramberg (1947) give in detail the procedure for the RCA model, in which the projector lens is fixed. Voltage centration is essential if resolution better than 40 Å is sought, in conjunction with correction of the objective polepiece for astigmatism.

6. Lens Correction: Through-Focal Series

The polepieces of any objective lens show a degree of astigmatism, no matter how well machined, owing to minute irregularities of bore and non-uniformity of the iron. The result is to limit the resolution to about 50 Å, even with an aperture of 10^{-3}. It is possible to correct this aberration by fitting screws of soft iron radially in the polepiece gap, and adjusting their distance from the axis until they produce a field in opposite orientation to that giving the astigmatism. The method is tedious, demanding careful observation of the diffraction fringes formed around opaque particles, such as carbon black, when the beam aperture is very small. Uniformity in width of the fringes is the criterion of correction. By systematic work the astigmatism, as measured by the separation of the two focal lines (cf. Section IV, 4b), can be reduced to about ¼ micron, compared with the 5 microns of an average untreated polepiece; this would be equivalent to a resolution of a few Ångstroms, if no other aberrations were present.

Most makers now supply objective lenses already fitted with screws and corrected. Those wishing to carry out the correction themselves will find full details given by Hillier and Ramberg (1947). In any case it is valuable to learn it, as correction is valid only for a particular voltage and magnification, and must be adjusted when conditions are changed. Also it needs checking after a few weeks, since hysteresis may produce changes in the iron. The most important part of the procedure is the evaluation of the astigmatism from the fringe width, as shown with typical micrographs by Hillier and Ramberg. The picture must be very close to exact focus for this purpose and the ordinary fine focus control is too rough for such accurate setting, even when a viewing telescope is used. A ten-times finer rheostat should be fitted in the objective lens circuit. By observing the ratio of each control to the next finer, and reading with a meter the current change corresponding to one division on the coarsest, the value of a division on the other controls can be found; for the finest it must be of the order of a few microamps.

Even so it is impracticable to work accurately enough by eye when focusing, and a through focal series has to be taken. The true in-focus setting can be found very closely from the circumstance that image contrast is then a minimum; on either side of focus Fresnel fringes form around the image, thus increasing contrast. After setting the finest control just short of this point, a series of five or six exposures is taken of the same particles, moving the knob by one or two divisions each time, depending on its range. In the negative it should be possible to see that the fringe width first decreases and then increases again; it is only by luck that an absolutely in-focus picture will be obtained, showing negligible fringes. Typical pictures in the paper quoted above will serve as a guide. It is usually found at first attempt that one has either started too far off focus, or changed the control in too large jumps. If too far off focus, the fringes may be so big as to hide the astigmatic effect and lead to the false conclusion that the lens is perfect. Once the near-focus image has been obtained, a systematic procedure of measuring the fringes and deducing which screws to adjust leads to correction by successive approximation. If a lens shows an astigmatic focal separation of more than 10 microns it is too bad to warrant fine correction and it is better to start with a new polepiece.

If an objective aperture is used with a corrected lens, even greater care than usual must be taken in cleaning it. A very small unsymmetrical deposit on it will introduce more astigmatism than the correction has taken out. It may be preferable to correct the lens without an aperture, and to use it subsequently in the same way, in spite of the poor contrast. The condenser must then be well defocused to give a small aperture and the alignment of the illuminating beam becomes very critical. If the beam makes an angle with the objective axis appreciable greater than its own angular aperture, the aberration for off-axis points may be considerable (Glaser and Grümm, 1952).

IX. Specimen Technique: Mounting and Shadowcasting

1. Support Grids

The standard object support is a metal grid of 200 meshes per inch, usually in the form of a disc of ⅛-inch diameter. Woven stainless steel or electro-deposited copper are most used, the latter being preferable owing to their greater stability when heated by the beam. Copper grids are now obtainable with a center diamond and pattern of bars that makes identification of squares easier. The Siemens model employs also a metal disc 3 mm in diameter and 1-mm thick, pierced with one or seven holes of 70-microns diameter; the Philips model uses a single slot in a silver plate, 1-mm long and 100-μ wide.

Grids should be stored in tubes or pillboxes so that they do not accumulate dust or get corroded. If there is any doubt about their cleanliness they should be rinsed in ether or re-distilled carbon tetrachloride. It is possible to re-claim grids after use, but great care has to be taken to remove all traces of specimen and support film; this is particularly difficult because the electron beam reduces the normal support film to an insoluble carbon or graphite. Usually it will not be found worth the trouble of re-claiming grids of copper or stainless steel. Those of platinum (Siemens) are more resistant and may be cleaned with strong acid or by flaming.

It is not difficult to prepare grids to any pattern desired, by a special photographic process followed by electroplating or a chemical etch. For instance a large mesh size may be required, or a large single aperture in the center of a fine grid. Full details of a straight-forward method are given by Challice and Sutton (1952).

2. Support Films

Some specimens, such as metal oxide smokes, carbon blacks and even diatoms, can be collected directly on the support grid. But usually a supporting film is necessary, which may be of collodion (nitrocellulose) or Formvar (polyvinyl formaldehyde). For special purposes films of beryllium, aluminum oxide or even glass have been used; details will be found in Drummond (1950) and Cosslett (1951). Here only the preparation of the two most generally used films will be described. (See also Revell and Agar, 1955.)

a. Collodion Films

The support film must be very thin, in order not to obscure detail in the specimen to be placed on it. A thickness of 150–200 Å is found to provide an adequate combination of transparency with mechanical strength; it should also be reasonable uniform and free from holes. Nitrocellulose films have the desired characteristics and are easily prepared. The simplest method is to take up in a fine pipette a quantity of a 2 % solution of nitrocellulose in amyl acetate and deposit a drop on the surface of clean distilled water contained in a deep Petri dish or glass bowl. The solution spreads rapidly across the water surface, the solvent evaporates and a thin collodion film remains. The first film prepared should be treated as a broom and discarded with the surface dust it collects. The second film is prepared for microscopy by dropping on to it the metal support grids, cutting round them with a fine needle, and lifting them off plus film on a washer fixed at the end of a metal rod. Alternatively, an area of film may be removed with such a washer and transferred to a grid supported on a metal pedestal.

Calculation will show what thickness of film is to be expected from a single drop of 2 % nitrocellulose spread uniformly over the available water surface; a 200 Å film would need an area of about 300 sq cm. In fact it is found that the film is thinner near its periphery and rarely reaches to the wall of the container, unless the surface is exceptionally clean. It is desirable to choose the strength of collodion solution in relation to the surface area so that a single drop gives a film of the required thickness. It is difficult to get anything like a uniform film from several drops, since they successively travel less far from the center. A little experience will show the correct strength of solution to give a single drop film of required thickness. It will also be found that some grades of nitrocellulose yield better films than others.

b. Formvar Films

Formvar is insoluble in solvents that spread well on water, so that films are made from it by stripping from glass slides. A 1 % solution in dioxane or chloroform is poured over the slide, or alternatively the slide is dipped into the solution, withdrawn and stood on end to drain. When the solvent has evaporated, the slide is slowly immersed in water at an angle of about 30° to the surface, when the film should come away and float on the surface. If stripping does not start automatically on immersion, the edge of the film may be teased off the slide with a needle. It usually helps if the edge of the slide (not the flat) is first scraped with a knife to remove adhering formvar.

The floating film is then cut and picked up as in the case of collodion. The film will not be uniform, but considerably thicker at the edge to which the solution drained. Interference colors will show the thinner (darker) region. If the film is found on examination to be full of holes, the indication is either that the slide was not dry before spreading the solution on it or that it was breathed upon before it dried. On occasion, a network film may be useful as a support and the best means of making it has been discussed by Schuster-Jaffe (1948).

If the slide used is new, no cleaning other than a wash in ether or dioxane is needed before applying the Formvar solution. If it has been used before, it should first be cleaned with dioxane, preferably in a fume cupboard, owing to the toxic character of dioxane, then flamed and finally washed in a pure solvent. Formvar films are distinctly tougher than those of collodion, and are recommended whenever heavy particles are present in the specimen or when subsequent washing or staining procedures have to be carried out. Collodion films can be stripped from glass in the same way, and are also found to be stronger than when cast on water.

It needs to be emphasized that the support films should be free of extraneous particles or bacteria which may be confused with the specimen itself. Solvents should be as pure as possible and preferably re-distilled. All containers, droppers, and tools for handling films should be well washed beforehand. The support grids should be kept in tubes or otherwise protected from dust and the water surface or glass slide for film preparation should be kept covered until actually used. Whenever there is doubt, blank films should be examined in the electron microscope for cleanliness. In industrial districts it may be necessary to arrange a dust-proof chamber for film preparation, or better still an air-conditioned room. Bacteria are liable to turn up even in distilled water, so that special care is necessary in preparing films for bacteriological work; specially re-distilled water must be used and frequently renewed, since bacteria may be introduced from tools or grids, or drop from the air on to the surface. When cultures are to be grown on the prepared specimen grid (cf. Section X, 1b) the grids themselves must be sterilized in ultraviolet light or chemically.

3. SHADOWCASTING

The method of depositing metal atoms on the surface of a specimen, from a directed beam produced by evaporation, is an invaluable means of enhancing the contrast of surface details. It was first perfected by Williams and Wyckoff (1944) using gold, but most other metals and many alloys have since been tried (cf. Williams and Backus, 1949; Hibi, 1952). Palladium, platinum, or a gold-palladium alloy are most used.

The principle is simply that the beam of metal atoms is caught by any projection on the surface, causing a drift on one side and a shadow on the other (Fig. 14). On viewing vertically in transmission a great accentuation of contrast is obtained in the surface relief, as in the case of a landscape seen from the air in the rays of the setting sun. Heavy elements must be used, so that a very thin layer gives enough contrast differentiation, since electron scattering depends on atomic number and density.

Several types of shadow-casting apparatus are now on the market, but are mostly expensive. As all that is required is a bell jar connected to a vacuum gauge, a diffusion pump, and a rough backing pump, it is not difficult to make a suitable evaporating plant with quite modest workshop facilities. A suitable design, constructed in this laboratory, is shown in Fig. 15; another is given by Wyckoff (1949, p. 83). The diffusion pump should be of high capacity (~ 100 liters/sec) and the bell jar no larger than absolutely necessary, so that pumping speed is as high as possible; a vacuum desiccator serves very well. The chief difficulty lies in sealing the current leads through the metal plate carrying the bell jar, but several methods are described in the literature (e.g., Herne, 1946). It is advisable to have two or three pairs of leads, so that successive evaporation of different metals or of the same metal from several directions may be carried out. It is in any case convenient to reserve different pairs for evaporating different metals, since some require a boat or basket and others simply a V-shaped filament.

Most of the useful metals have high evaporating temperatures so that an even higher melting metal must be used for heating it. A V-shaped tung-

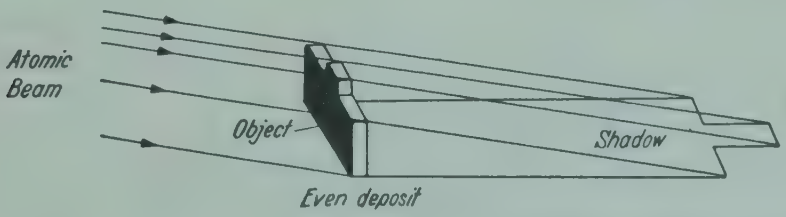


FIG. 14. Schematic diagram of metal-shadowing technique.

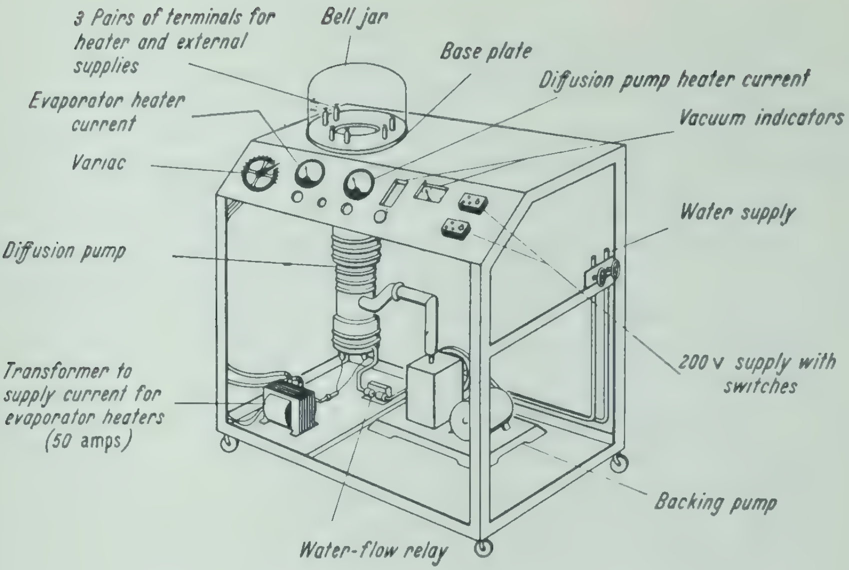

3 Pairs of terminals for heater and external supplies
Bell jar
Base plate
Diffusion pump heater current
Vacuum indicators
Evaporator heater current
Variac
Water supply
Diffusion pump
Transformer to supply current for evaporator heaters (50 amps)
200 v supply with switches
Backing pump
Water-flow relay

FIG. 15. Sketch of plant for metal shadowing.

sten filament is most generally useful, made of wire 0.2-0.5 mm in diameter. It should be kept as short as possible between terminals, and will require 15-30 amperes from a step-down transformer, according to the metal to be evaporated. A small boat of tantalum is suitable for powders such as beryllium. It may be made very simply by punching an indentation in a 2-mm wide strip of fine gauge, or by making a longitudinal crease in the strip. For chromium and other metals in the form of chips, and also silica for replica work, a small basket of tungsten wire is made by winding it around the conical end of a large wood screw and filling the gaps with alumina paste.

The simplest geometrical arrangement of source and specimen is that in which the prepared grids lie horizontally on the base plate of the bell jar at a distance of 10–15 cm from the filament, which is raised to a height appropriate to the shadowing angle needed. Alternatively, and especially when powders are evaporated from a boat, the grids may be held on a glass slide with spring clips of brass or phosphor bronze, and this in turn clamped above or opposite to the filament on an arm, the angle of which can be varied. A separation of at least 10 cm is desirable to protect the specimen from the effects of heat and to give a sufficiently uniform distribution of deposit over a row of grids.

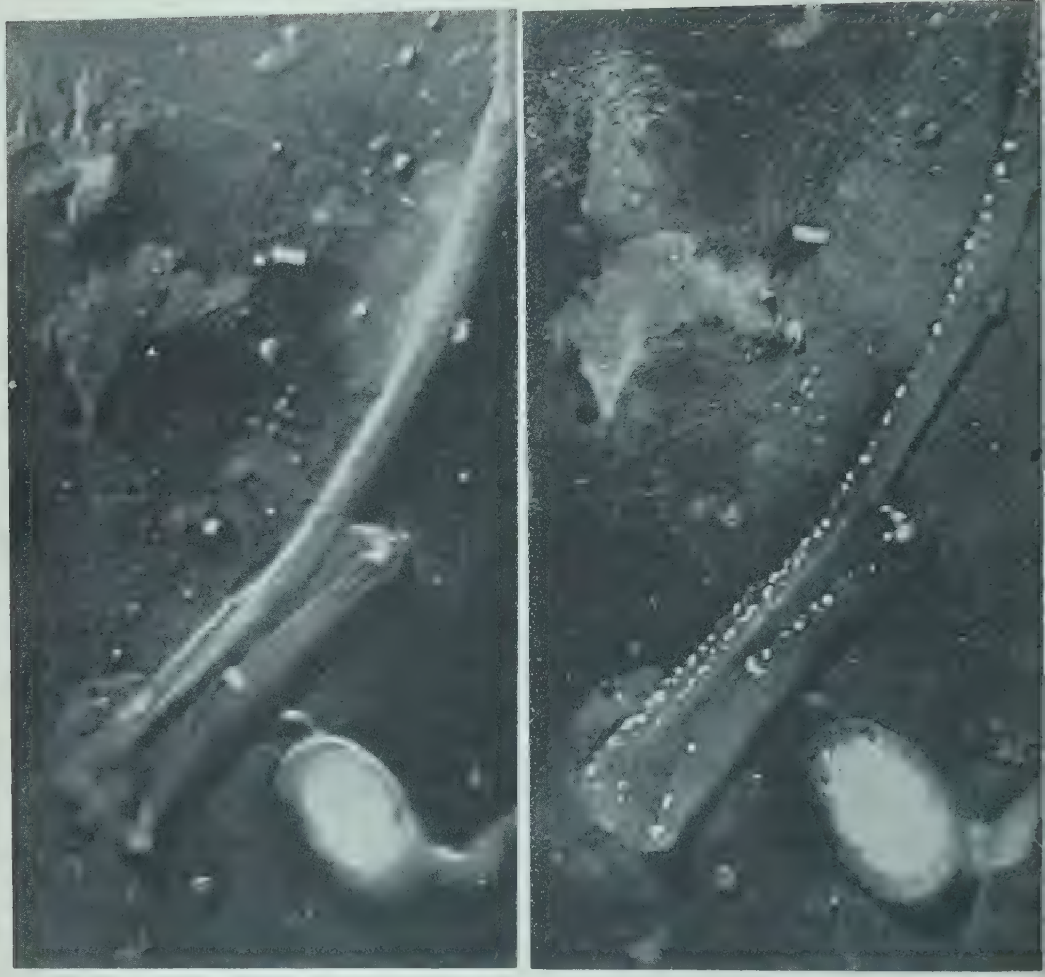
An angle of 20–30° is suitable for the ordinary run of specimens, often approximately specified as $\tan^{-1} \frac{1}{3}$ or $\frac{1}{2}$ respectively. Shallower angles give long shadows which may blanket important detail and also pile up too much metal on the "windward" side of projections. When the features of interest are all very small, as with plant viruses or macromolecules, it may be useful to use an angle of 10 or 15°, but care must then be exercised in calculating particle size since the deposit may be of appreciable relative thickness. For all ordinary purposes a deposit of thickness 10-20 Å only should be used. Assuming spherical symmetry of evaporation, which is roughly true for a deposit at some distance from a filament, the formula for calculating the thickness t over a surface inclined at an angle θ to the atom beam is

$$t = \frac{m \sin \theta}{4\pi l^2 d} \tag{7}$$

The thickness on a projection that is normal to the beam is given by omitting the term $\sin \theta$. Here d is the density of the metal used, l the distance of specimen from filament, and m the mass of metal evaporated. A 20 Å layer on a surface at 20° at a distance of 10 cm would need 15 mg of platinum (density 21.5) or 11.5 mg of 60 % gold-40 % palladium (density 16.5). In practice evaporation is not symmetrical nor completely efficient, and the layer may be thinner than calculated. To avoid troublesome weighings, it is convenient to obtain the metal in the form of wire of given mass per unit length so that a few millimeters may be cut off as required. Wire is also easily wrapped around the heating filament—which should be slowly raised in temperature so that the wire does not melt too rapidly and partly fall off.

The chief difficulty in shadowcasting is the tendency of the deposited metal to aggregate into small crystallites, which may be mistaken for particles in the specimen. Even if this is avoided during evaporation, it may occur in the electron microscope if the illuminating beam is too intense (Fig. 16). The remedy is to choose a metal that shows minimum granulation and to take precautions in shadowing by using only a thin layer and depositing it in the highest vacuum conveniently obtainable. Nickel, platinum, and palladium are recommended, pure gold and chromium are not, although it is claimed that chromium gives a good uniform layer if thinner than 10 Å. Alloys naturally show less tendency than pure metals to form crystals. Gold-manganin wires twisted together give good results; in this laboratory gold-palladium alloy (60–40) is usually used, being obtainable as prepared wire of convenient thickness.

The degree of vacuum is of great importance. At least 10^{-4} mm Hg should be aimed at, and better for high resolution work. It is not sufficient to pump until this vacuum is nominally obtained according to the gauge, since residual oil and other vapors are desorbed only very slowly from metal surfaces. It is advisable to pump for at least half-an-hour, and improvement in uniformity of deposit is usually found up to an hour or more. It also seems



(A) (B)

FIG. 16. *Euglena gracilis* flagellum (A) before and (B) after exposure to intense electron beam, showing granulation of shadowing metal (Cavendish Laboratory), ×18,000.

to be of value to install a shutter between filament and specimen, so that the latter is exposed only for the minimum time, which can be controlled; a nozzle or jet for directing the shadowing beam has also been recommended (Hibi, 1952).

It is usually sufficient to shadow once only at a given angle and direction, but Drummond (1950) has recommended using two successive beams from different directions to show the shape of particles, and this method has been developed into a method of "portrait" shadowing by Philpott (1951). Heinmets (1949) has gone so far as to rotate the specimen during shadowing to increase contrast all round, but this seems to be of restricted applicability.

The chief reservation about shadowing is that it should not be used when an indication of body distribution of matter in the specimen is sought, as in studying developmental stages of bacteria (cf. Fig. 17). It should really be reserved for cases where the primary interest is in surface relief, in spite

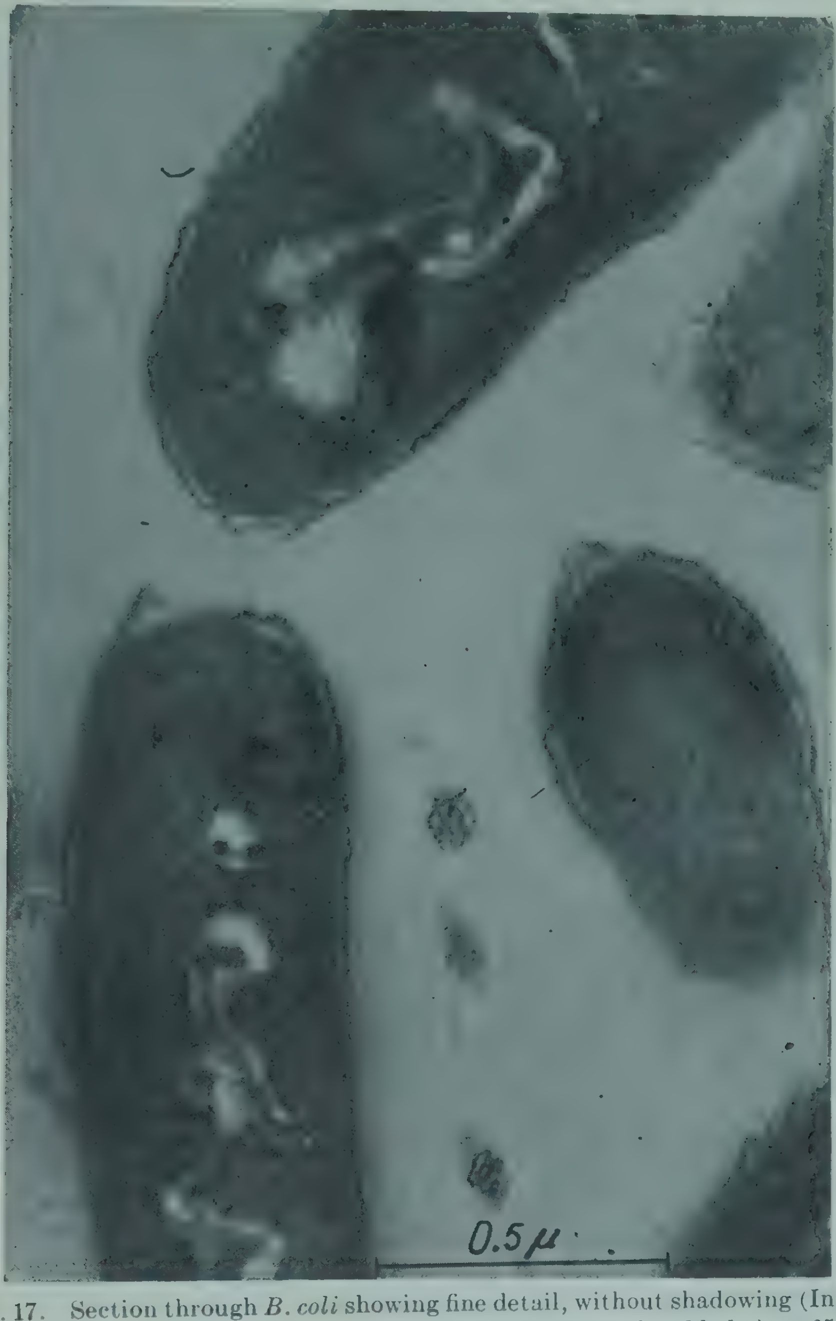


Fig. 17. Section through *B. coli* showing fine detail, without shadowing (Institute of Public Health, Copenhagen, and Karolinska Institute, Stockholm), ×65,000.

of the great temptation to get show pictures by its use. In many cases where it might add slightly to the pictorial value, the time consumed in properly shadowing makes it uneconomic, especially in routine runs. There are cases in replica work where it is of value, but if the replicas are properly prepared the contrast should already be adequate for most purposes (cf. Section XI). Usually the resolution is limited by the replica itself, and is not improved by shadowing; the main exception is the stripped replica, in which shadowing is an integral part of the process. Wyckoff (1949) also recommends shadowing as a means of electrostatically stabilizing sections and other specimens, which may have thick regions which absorb a considerable number of electrons from the beam.

X. Specimen Technique for Biological Material

1. Particle Suspensions

a. Inorganic Material

Many substances with particles of colloidal size may be of biological interest, e.g., on account of their effect on human beings or experimental animals. They may be aerosols, such as mine dusts, or in liquid suspension, as with clays or pigments. In either case they are simple to transfer to filmed microscope grids for examination.

Airborne particles may be simply collected on grids exposed in the airstream maintained by an aspirator. The use of a thermal precipitator specially designed for electron microscope work has been described by Walton (1947). Considerable care has to be taken to ensure that a properly representative sample is collected, if a detailed particle size distribution is to be obtained.

Liquid suspensions are directly applied to the grid in small drops from a pipette. The concentration needed to give a reasonably covered field in the electron micrograph may be roughly judged from the opacity; it should be distinctly milky, but still translucent. The exact concentration will vary, of course, with the size of the particles. For vaccinia or Dow latex, of diameter about 0.25 μ, 0.2–0.5 % is suitable; for much smaller particles, such as plant viruses, greater dilution is needed. Since particles of the order of a micron or larger absorb too much energy from the electron beam and disrupt the film, and also may obscure the field unduly, it is often necessary first to remove them by levigation or centrifugation.

Aggregation sometimes causes difficulty and may be overcome either by trying other dispersing agents, by varying the pH, or by sonic or supersonic vibration. Both Drummond (1950) and Schuster and Fullam (1946) give details of dispersing procedures specially suited to a variety of powders. For mechanically shaking suspensions it is possible to buy commercial vibrators, but it is equally satisfactory to use a loud speaker with the axis of the cone vertical and a light holder for the tube of liquid.

b. Bacteria and Viruses

In general bacteria and viruses are treated in the same way as inorganic suspensions, and deposited on filmed grids as small drops from a sufficiently high dilution to give well spaced individuals in the high magnification image. But additional difficulties are liable to arise in getting a clean preparation and in preventing aggregation, partly on account of the organisms themselves and partly from the fact that they have to be cultured on media containing material inimical to microscopy, such as inorganic salts or glutinous material.

It is preferable to fix the organisms either directly on (or in) the growth medium or immediately after removal from it, to minimize subsequent damage during preparation. In general 5% formal or the vapor of osmium tetroxide gives best fixation, the latter giving more contrast owing to its high atomic number (cf. Section XII, 1a). From semi-solid media bacteria may be washed off in distilled water or formalin, and are often clean enough for immediate examination in the electron microscope. Otherwise they are spun for a few minutes at 3,500 rpm, re-suspended in distilled water and if necessary spun once more.

The final suspension is best transferred to the prepared grid by means of a platinum loop of 1-mm diameter, which can readily be sterilized by flaming. Ease of spreading is aided by adding a drop of detergent such as saponin. For giving a uniform and clean sample, Hartman *et al.* (1953) recommend making up the suspension with a protein such as serum albumin and spreading on the surface of water in a special Langmuir trough. Kellenberger (1949) has devised a method of depositing bacteria directly on to grids by "microcentrifugation."

Cultures in liquid media can be purified from extraneous matter by a similar procedure. Alternatively, it has been found adequate for many purposes to drain away foreign matter by simply placing a drop of uncentrifuged suspension on the filmed grid, pricking a hole near the center of the film and allowing the fluid to drain through into distilled water. Good clean preparations of various bacteria have been obtained by this method (Valentine and Bradfield, 1954).

It is also possible to grow bacteria directly on filmed grids, with the great advantage that they can be fixed *in situ* and examined with negligible disturbance of their mutual arrangement (Brieger and Cosslett, 1949). Alternatively a collodion film can be spread over the whole culture medium, inoculated and incubated for a given time; the film is then floated off on water and selected portions transferred to grids for examination (Hillier *et al.*, 1949). The first method has the advantage that sample growth can be followed continuously from a single inoculation. It is also easy to sterilize the filmed grids before depositing them on the medium, either in an autoclave or by ultraviolet irradiation. However, it may be that the presence of copper has an inhibitory effect on the growth of some organisms; it is better to use stainless steel or silver grids. No appreciable effect on the rate of growth of *B. coli* has been found on filmed grids, compared with normal culturing procedures on semi-solid media; direct comparison can be made by the impression technique. For liquid media the filmed grids are placed face down on filter paper floating on the surface, or placed on pads of cotton-wool soaked in the liquid (Brieger *et al.*, 1953).

With viruses the major problem is to obtain a reasonably pure specimen, free from cell debris and normal cell constituents. In the case of the larger viruses, such as the vaccinia group, direct centrifugation is usually adequate; aggregation may be countered by sonic vibration or change in pH. Suspensions of smaller viruses often contain much other matter, even after ultrafiltration. Simple washing removes much of it: a drop of virus suspension is placed on the filmed grid and before it is quite dry a drop of distilled water is deposited on it, left for a few seconds, and then removed with a micropipette or with filter paper. The virus particles appear to be absorbed firmly to the collodion film, so that mainly debris is removed by washing.

It is not possible here to detail special methods that have been found useful for particular viruses (cf. Vol. II), but one or two are of more general use. For instance, it has been found that influenza and some other viruses are firmly absorbed on to laked ghosts of red blood cells (Dawson and Elford, 1949). The loaded membranes are isolated by mild centrifugation, and then viewed directly in the electron microscope if few particles are present; alternatively the virus bodies may be removed from the membrane by chemical treatment or by sonic vibration, and deposited from suspension in the usual way. The method of Fulton and Begg (1946) for purifying rickettsiae has been found useful also for mouse pneumonitis virus by Cosslett *et al.* (1950). After differential centrifugation, tissue debris is removed by absorption on kieselguhr; some virus particles also are lost by absorption, however. More detailed information on methods of preparation is given by Wyckoff (1949), for plant as well as for animal viruses.

c. *Larger Microorganisms*

The methods used in purifying and mounting bacteria are mostly applicable also to larger organisms, such as protozoa, spirochetes or spermatozoa. Repeated centrifugation is often necessary to obtain a clean preparation; a method which has been found useful is as follows. Dilute 0.5 ml of the concentrated suspension (e.g., semen) with 10 ml of physiological saline or other suitable medium, such as phosphate buffer. Centrifuge at a speed just sufficient to bring down most of the organisms (~ 1000 g) for 5 to 10 min, decant thoroughly and re-suspend in 5 ml of saline or buffer. Add 5 ml of 4 % formal-saline, stand for 10 min and centrifuge again at moderate speed; clumping will result if spun too hard. Decant thoroughly again and re-suspend in fresh distilled water; if necessary, spin, decant and re-suspend once more. The final concentration should be such as to give about 5 organisms per mesh on the support grid, when a small drop is applied with a platinum loop.

Organisms of this size may absorb so much energy from the electron beam that the supporting film breaks. Thicker Formvar films may prove strong enough, or aluminium oxide films electrolytically prepared. Benefit is also claimed from exposing the film beforehand to an electron beam of moderate voltage (1000 v).

2. THIN SECTIONS

It has not proved so difficult as was initially supposed to cut sections of tissue thin enough for electron microscopy. Compared with the minimum thickness of 1 micron in optical microtomy, sections must be no thicker than 0.2 micron and preferably 0.1 micron or thinner. As a rough rule, the thickness should be no greater than ten times the resolution sought. With a microscope capable of 30–50 Å, sections as thin as 300 Å are desirable and have now been regularly obtained in several laboratories. Generally speaking, adequate results can be obtained from almost any type of knife and any type of microtome, suitably adapted; the major difficulties of preparation and interpretation are concerned with fixation, embedding and dewaxing of the tissue.

a. Fixation and Embedding

As discussed later (Section XII, 1a) there is now considerable evidence that buffered osmium tetroxide gives best fixation, although with many tissues formalin gives almost identical results. It is good practice to try both fixatives, and to compare the results with freeze-drying whenever there is any doubt about the structures observed. Embedding may be done with some volatile fixative, such as naphthalene or camphor compounds (Fullam and Gessler, 1946), or with normal materials. The former have the advantage that they volatilize in the electron microscope and give a more transparent section, without solvent extraction, but artifacts may be caused by over-rapid sublimation of the material. With the increasing need to cut very thin sections, general use is being made of hard waxes, such as ester wax, and of methacrylate compounds (Newman *et al.*, 1949; Wyckoff, 1949). Some workers have used double embedding, with wax and celloidin, with the advantage that the wax may be dissolved out, leaving celloidin to hold together the tissue. Removal of methacrylate with amyl alcohol is possible, but only at the risk of disturbing the material in the section; comparison with unextracted sections is essential in such cases. When wax is employed, it is usually found that much of it evaporates in the vacuum of the electron microscope, especially under very weak electron bombardment; a stronger beam "fixes" the wax, probably by carbonization or polymerization, and renders it non-volatile and unextractable by solvents.

It is convenient to use much smaller blocks than in optical microtomy, owing to the size of the specimen support, and a cube of 1 mm edge is adequate. Embedding is then rapid, even if carried out at a slightly larger size with later trimming of the block.

b. *Sectioning*

As stated, almost any standard microtome can be adapted for thin sectioning by changing the advance mechanism and knife support (cf. Pease and Baker, 1948; Bretschneider, 1949). Other workers have built special cantilever (Cocks and Schwartz, 1952), rocking (Danon and Kellenberger, 1950), and wheel (Sjöstrand, 1953) mechanisms to give rigidity and repetivity. The simplest method is to dispense with mechanical advance altogether, relying on thermal expansion of a rod to move the block (Newman *et al.*, 1949). It is desirable to have a rotary or osculatory motion such that the block does not pass the knife on the return stroke.

The knife itself may be a normal microtome blade specially sharpened (cf. Hillier, 1951; Sjöstrand, 1951) or an ordinary safety razor blade without special sharpening. It must be held rigidly in two clamps, which are close together, since only a small cutting length is required. The cutting angle is not critical nor the temperature of sectioning; the cold conditions sometimes recommended seem to be unnecessary if a very slow rate of cutting is adopted (2–4 seconds per section). It is desirable not to breathe on the block during cutting nor to have lamps close to it, since a very small temperature rise can give undue expansion. It is helpful to watch the process of sectioning through a low-power microscope (×20). It is almost essential to fix a small waterbath to the knife edge, so that the meniscus of the liquid comes up far enough to draw away each section as it is cut; serial sections float out on to the surface and may be picked up on wire gauze or directly on specimen grids. An anti-rusting agent such as sodium chromoglucosate should be added to the water. Some workers prefer to remove the sections from the knife with a fine brush, and flatten them on 50-50 dioxane-water at 40°C. Knives of glass can also be used (Latta and Hartmann, 1950), in the form of a lozenge with the sharp edge (~ 30°) disposed for cutting. Plate glass about ¼-inch thick can be readily cut into such pieces, and those of best cutting edge selected; only a small part of the edge is usually straight enough to use. They have the advantage of being cheap and of avoiding the re-sharpening associated with ordinary microtome knives; it is also easy to fit a small water-trough to them. However, they have a short cutting life and appear to have no advantage over a good safety razor blade. It is sometimes stated that glass knives are essential for cutting methacrylate embedded specimens, but this is not the case.

Most of the commercially available microtomes for electron microscopy will cut sections thinner than 1 micron—usually down to 500–700 Å. Some specially built models have cut as thin as 200 Å(Fig. 17), in which case it is unnecessary to remove the embedding material (cf. Sjöstrand, 1953). It thus can be said that the demands of electron microscopy can be satisfied, at least up to the present-day resolution for biological specimens. Discussion of artifacts is deferred to Section XII.

3. STAINING

In optical microscopy colored dyes are used to make visible features of the specimen that are translucent in ordinary light. The corresponding staining procedure for electron microscopy is akin to that practiced in x•radiography: the selective absorption of compounds of high scattering and absorbing power for electrons. Thus substances of high atomic number must be sought, which normally implies great physical density also, such as gold, lead, and tungsten compounds. Little systematic investigation has yet been conducted to find what substances may be specifically taken up by different biological features, apart from the testing by Richards (1952) of a variety of compounds on insect cuticle. Such work as has been published shows that most heavy elements such as lead salts or osmic acid are too generally absorbed to be of value. The staining of the bands of muscle and collagen by phosphotungstic acid (Hall *et al.*, 1945; Gross and Schmitt, 1948) is the main positive result so far recorded. The process of treatment is to soak the dissected fibers for 1 min in a solution of 0.1 % phosphotungstic acid and a buffer of potassium acid phthalate and NaOH (pH 5.1 to 5.5). They are then removed, washed, and mounted on the support grid.

There is undoubtedly a need for more extensive trials; much may be learnt even by trying standard optical staining techniques, or variations of them, since they often appear to depend on a physical aggregation of material as much as on staining in the strict sense. Now that very thin sections can be cut, the way is open for a gradual accumulation of experience on the best methods of treating each type of tissue, as has occurred in optical microscopy. In the process much may be learnt by comparative study of the artifacts produced by optical and by electron microscopical preparative methods, in fixation and drying as well as in staining.

XI. Replica Techniques for Biological Material

Methods of replicating surfaces have been mostly worked out for metallurgical specimens, which are usually too thick for direct electron microscopy and from which thin sections cannot be made. Some of these methods are applicable to biological material, and one or two new processes have been specially developed for the purpose, particularly for dealing with wet or porous surfaces. The end result of any such method is a replica in the form of a thin film of plastic or metal, which can be examined in the electron beam. It may be obtained either at first-hand or second-hand from the original surface, being known as a negative or positive replica respectively. The latter will be most readily interpreted, having the same contrast scheme as the original, but involving a two-stage process; it is especially valuable when the first replica can only be made in a relatively massive block of material. Three-stage processes have also been devised for special needs, such as porous surfaces.

1. Single-Stage Replicas

a. Formvar Film

The simplest way of making a replica of a surface is to paint it with a weak solution of a plastic material. The film which forms on drying can often be stripped intact and mounted for direct examination in the electron microscope. Thick parts of the film correspond to depressions in the original surface, thin parts to elevations, so that the electron transmission is inversely proportional to surface height. The micrograph negative thus reproduces the correct contrast scheme of the specimen, the final print giving reversed contrast (Fig. 18).

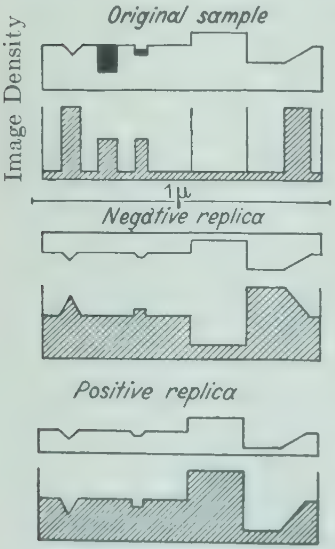


Fig. 18. Formation of replica films and corresponding contrast in image.

The most favored replica material is Formvar in 1 % solution in dioxane or chloroform; experience shows how much to apply to a given surface area to yield a film thin enough for examination yet thick enough to stand stripping. It may be removed in some cases by simple immersion in water, as when stripping support films off glass. More usually it is backed by cellulose tape or a thicker film of another material (such as gelatin), stripped and then released by immersion of the composite film in a suitable solvent for the adhesive (e.g., acetone). With flat surfaces it is possible to use the technique devised for metals, in which the support grid is first stuck to the Scotch tape with a piece of cigarette paper to protect its center, pressed over the selected region on the surface, and the replica stripped direct on to the grid, which can be subsequently cut off the tape.

The direct replica method can be used on dry surfaces that are reasonably flat, such as teeth and bone. It becomes difficult to detach it from more rugged areas. Methyl-, ethyl-, and nitrocellulose have been used as well as Formvar, the first being water soluble.

b. Pre-shadowed Replicas

When the investigated surface can be placed in vacuo, a replica can be made by evaporating a film on to it. Most metals cannot be readily removed from biological material, but silicon monoxide has been employed successfully (Hass and Scott, 1949) (Fig. 19). The method is more often used for forming a thick first replica (see below) or for putting down a shadowing layer of metal which is subsequently stripped by backing with thin nitrocellulose or Formvar. This "pre-shadowed" replica method combines the advantages of shadowcasting with replication (Williams and Wyckoff, 1946) and has been developed by Wyckoff (1949) for several biological purposes,

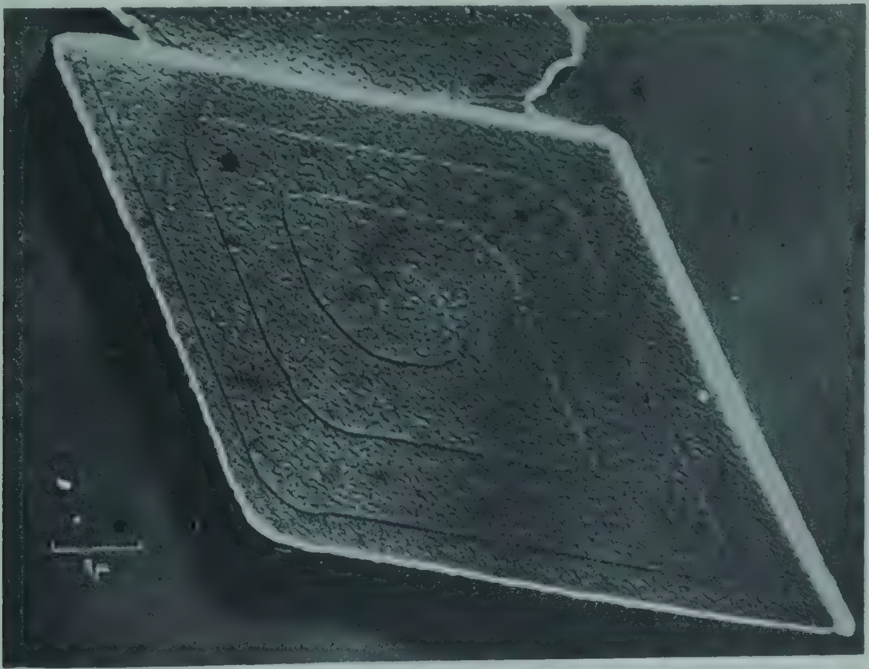


FIG. 19. Silicon monoxide replica of fatty acid crystal (Glasgow University), ×10,000.

more particularly for showing the crystal structure of plant viruses (see also Reed and Rudall, 1948).

c. *Carbon Films*

A rather different type of replica is obtained by exposing the specimen *in vacuo* to an electrical discharge at about 600 v in an atmosphere of benzene or toluene (König and Helwig, 1951). A "contaminating" layer of carbon is rapidly formed over the surface, from which the carbon film can be subsequently removed in a suitable solvent, leaving the film on the surface of the liquid. Such a replica is almost structureless and so gives a higher resolving power than plastic films. It also allows replicas to be obtained from projections or pits, where a stripped replica would tear away. The film can also be evaporated from a carbon arc (Bradley, 1954).

2. Two-Stage Replicas

a. *Polystyrene-Silica*

The first replica may be made by painting the surface with a lacquer of polystyrene dissolved in benzene (Gerould, 1947). Alternatively a more massive block of polystyrene may be pressed on to the surface, at about 1 kg/cm², and softened by raising to 130°C, as advised by Barnes *et al.* (1946) for biological material. In either case the specimen normally has to be dissolved away in a suitable solvent or by enzymatic digestion. A silica layer is then deposited *in vacuo* on the surface of the first replica, and the latter then dissolved by immersion in ethyl bromide; a description of a simple apparatus for the purpose is given by Drummond (1950). The method gives good contrast, but some difficulty is encountered in interpretation owing to the mobility of silica on the surface during deposition. Silicon monoxide gives stronger and more structureless films (Hass and Scott, 1949). The high temperature which is needed is also a disadvantage for biological work, as distinct from metallurgy. For this reason Kellenberger (1948) has used a thick nitrocellulose film wetted with acetone for the first replica, pressing it on the surface.

b. *Methacrylate-Metal*

Monomeric methacrylate derivatives will polymerize at room temperature and so allow replicas to be made without harming the specimen (Brown and Jones, 1947; Brown, 1950). Methyl methacrylate as used in dentistry is suitable, after removing the stabilizing hydroquinone by shaking with one-fifth its volume of 20% NaOH in a separating funnel and discarding the brown lower layer. It is washed twice with distilled water, running off the lower layer each time, and dried overnight over calcium chloride. Before application, polymerization is started by heating 5 cc of the liquid with 3 mg benzoyl peroxide for 30 min at 90°C. It is then applied to the specimen and left overnight to set. If the nature of the specimen permits, polymerization may be hastened by mild heat or by ultraviolet irradiation. When hard, the major part of the specimen can be peeled away and the rest removed with solvents or by digestion.

The final replica is made by evaporating chromium or other metal on to the surface to a thickness of about 100 Å. The metal film is then removed from the methacrylate by immersion in chloroform. To minimize tearing of the film it is as well to back it first with collodion or beryllium, in which case a thinner evaporated layer may be used. The resolution of these replicas is as good as that of the pre-shadowed or carbon methods, and has the advantage that it can be applied under atmospheric conditions.

c. *Silver-Carbon*

A method of replication using silver in the first and nitrocellulose in the second stage was devised by Zworykin and Ramberg (1941). An improved process has been used by Dalitz (1953). The silver is evaporated *in vacuo* over the prepared specimen to a thickness of 2 microns; about 0.5 g of

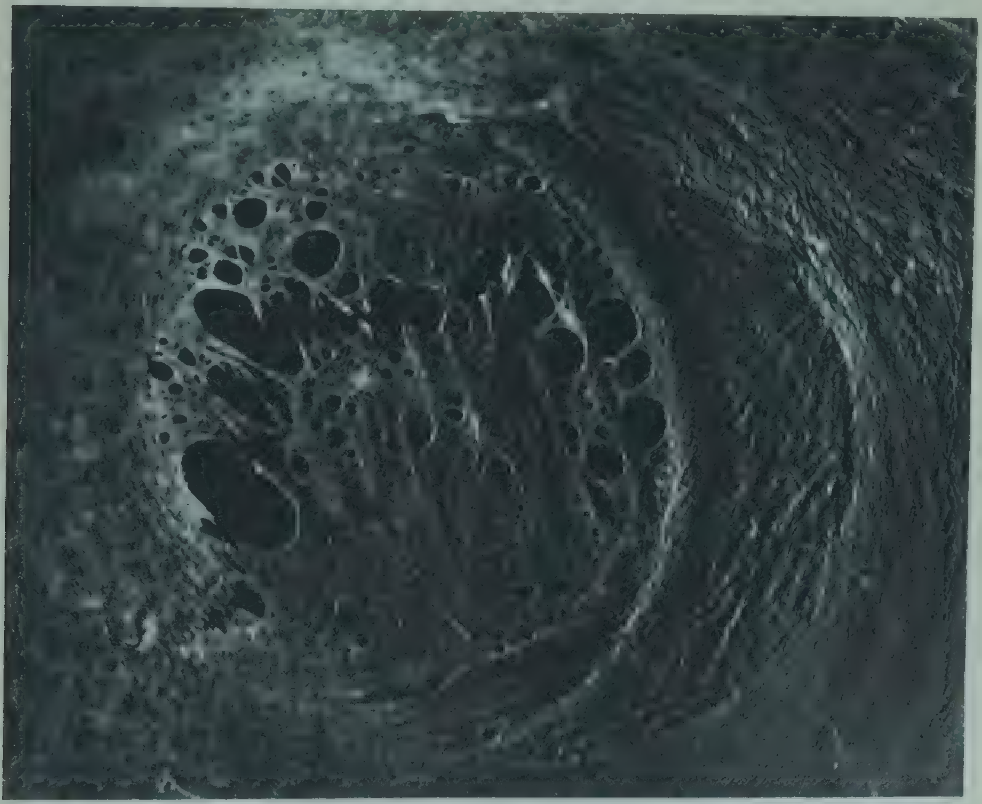


FIG. 20. Carbon replica of pine wood (Technical University, Delft), ×20,000.

silver is needed. This film can normally be peeled away, and is then mounted on a brass anode for exposure to an electrical discharge in toluene vapor (see p. 516 above). The resultant carbon film is detached by immersion in dilute nitric acid; it floats on the surface and can be picked up on the support grid. Excellent replicas of wood fibers have been made by this method (Fig. 20).

3. Replica Methods for Wet or Porous Surfaces

a. *Plastic-Metal*

The methacrylate method is applicable to surfaces which have been only superficially dried, the specimen generally remaining undesiccated. In this way Brown (1950) has obtained replicas of hemoglobin crystals, and Brown and Jones (1947) of other biological material in the wet state.

An alternative method has been used by Wolf (1944) using a synthetic plastic (Palapont) in chloroform, which does not form an emulsion with water; Formvar can also be used. The solution is applied to the specimen surface, and when dry removed with Scotch tape. Any residual material is cleaned from it, the tape removed in absolute alcohol, and a film of chromium then deposited *in vacuo* to form a positive replica. The film is scribed into squares and immersed in chloroform to dissolve the plastic, so that the chromium replica floats on the surface and may be collected on a support grid. Wolf used the method to obtain replicas of sections, as well as of bone, tooth and various tissues.

b. *Three-Stage Silica Replica*

It was found possible by Fischbein (1950) to obtain satisfactory replicas of wet surfaces of wood either directly in a film of polystyrene or polyethylene, or in a two-stage process with silica as the second replica. The first replica was made by covering the specimen with a very thin plastic film, clamping the two between glass slides and immersing in water at 100°C for 7 min. Better results were obtained, however, with a three-stage method in which the first replica is made as described, in a film of polyethylene 0.1-mm thick. A second replica is made by painting the first with a 1% solution of polystyrene in benzene, and stripped from it after backing with gelatin. The gelatin is removed in hot water and silica evaporated, after which the polystyrene is removed by reflux washing with carbon tetrachloride.

c. *Three-Stage Aluminum Replica*

Porous substances present special difficulty in detaching the first replica, but Varossieau (1949) has devised a three-stage method which overcomes it, at least for materials such as porcelain and wood. The first replica is made in a 3 mm block of alkathene (polyethylene) at a pressure of 1 kg/cm^2 and at a temperature of 115°C. A low viscosity polymer is preferred, and grade 200 was actually used (made by Imperial Chemical Industries). The replica is removed from the surface with a knife blade and coated with a drop of a 2% solution of nitrocellulose, followed after drying by a drop of 10% nitrocellulose as a backing film. The composite second replica is stripped and mounted in a vacuum chamber, where a film of aluminum is deposited to a thickness of 200-400 Å. Evaporation is best carried out with a multiple source, or with movement of source or specimen, to obtain a uniform deposit. The nitrocellulose film is removed with the reflux vapor of amyl acetate, and the aluminum third replica mounted on a support grid. When the original surface is not too rough one of the stages may be omitted, either by taking a thin nitrocellulose replica from the alkathene film, or by making a thick nitrocellulose replica of the original surface and an aluminum one from it as before.

d. Low Temperature Replica

A method for replicating aqueous substances in the frozen state has been described by Hall (1950). The specimen is carried on a glass slide placed on a copper block, pre-cooled to liquid air temperature, in a vacuum chamber. The temperature of the specimen is momentarily raised to − 60 to − 90°C by radiant heat in order to sublime ice crystals and to secure a surface etch. The surface is then shadowed with chromium followed by a supporting film of silicon monoxide from a crucible of beryllium oxide. The cold block is removed from the vacuum, and the replica film floated off the specimen by immersion in water, after warming to room temperature. Good results were obtained from ice crystals and from a frozen suspension of silver halide particles, but the method would appear to be of wider application.

XII. Artifacts

Electron microscopy sometimes requires even more interference with the specimen than is necessary in the normal mounting and staining methods of optical microscopy. It is therefore important to pay careful attention to the dangers of artifact production at the successive stages of fixation, dehydration, possibly sectioning and extraction, and finally of exposure to the electron beam. Evidence is beginning to accumulate on the type of damage that can occur, and on the whole it shows that the dangers are not so great as were at first thought. The best advice that can be offered is to employ several different methods of preparation whenever possible, and to compare the results one with the other and especially with optical studies. The phase contrast and ultraviolet microscopes are especially valuable for this purpose.

1. Artifacts of Preparation

a. Fixation

To some extent the lessons learnt with the light microscope on the effect of fixatives can be carried over into electron microscopy. But it is necessary to remember that the details sought with the latter are so very much finer, and the best optical fixative may not be best for macromolecular structures. In optical work fixation has to make features visible within the resolution limit of light, and this must often involve agglomeration or thickening of thinner features in the specimen. In the electron microscope we wish fixation to leave undisturbed detailed structures of the order of 100 Å or less. One cannot take over automatically the fixation techniques of normal microscopy without critical examination of their results at high magnification, and this work is now beginning. Preliminary results from Rosza and Wyckoff (1951) and from Palade (1952) indicate that the most suitable fixative for many tissues is neutral osmium tetroxide. The former workers showed that Flemming's solution and other acid fixatives caused changes in submicroscopic structure (Fig. 21). Formalin gave better results, but they varied according to pH and to length of fixation; the fine-grained structure of protoplasm was best preserved by fixation in 4% neutral formalin for 2 hours. These authors also prefer pyridine to alcohol for dehydration, prior to embedding for microtomy, as passage through alcohols of diminishing water content frequently causes undue shrinkage of the tissue.

Palade investigated mainly the effect of pH on fixation with osmic acid, using a variety of tissues. He found that the cytoplasm was most continuous and cell structures best preserved when 1% OsO_4 at a pH of 7.3–7.5 was employed and a veronal-acetate buffer. There appears to have been no comparison of such fixation methods with freeze-drying, which should

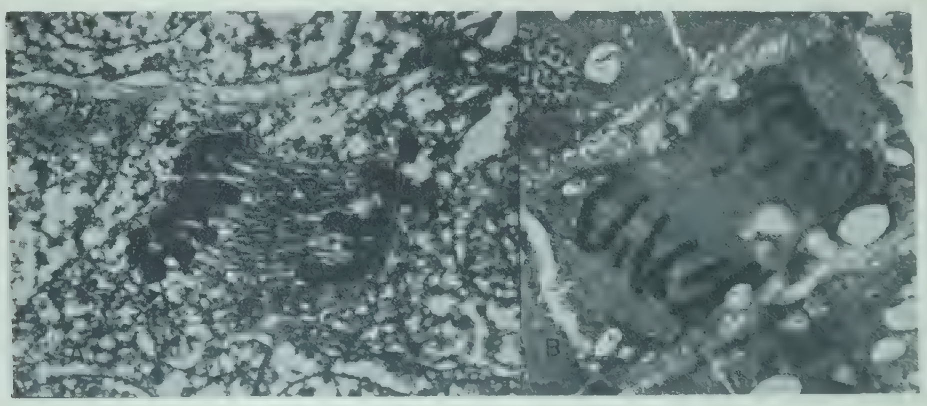


FIG. 21. Onion root tip cell in anaphase (A) fixed in Flemming's solution, and (B) fixed in formalin and shadowed. (National Institutes of Health, Bethesda.) ×2,000.

aid in forming an opinion as to the real appearance of submicroscopic structures. It is in general assumed that pronounced granularity of the contents of cell or nucleus, or thickening of membranes, is a sign of bad fixation, but there can be no certainty that these criteria are correct without a great deal more experience.

b. Drying

The most probable source of artifacts in electron microscopy is the desiccation essential to vacuum working. Since all biological material has a high water content, the shrinkage on drying is bound to be considerable. It will have two consequences: an overall reduction in dimensions of the specimen, and a disturbance of its internal structure, by displacement where there is differential shrinkage and possibly also by aggregation of material. The gross shrinkage can be estimated by comparison with ultraviolet and phase contrast pictures, although only on a statistical basis, since organisms usually vary considerably in size in a given population. For electron microscopy they are settled on to a supporting film, with the result that there is little shrinkage of the area of contact but great collapse in the third dimension. Even when fixed, bacteria will normally dry down to half their initial height, and without fixation to much less. It is probable that much re-organization of the interior structure takes place at the same time, leading to aggregation in some parts and the formation of relative vacuoles in others. Some check on the larger changes are available optically; for instance, the ordinary light microscope, and even better the phase contrast method, shows clearly the dense regions in mycobacteria (Brieger and Fell, 1945) which appear as granules or "polar bodies" in electron micrographs.

The small scale desiccation changes can now be checked also by thin section technique, in which it can be assumed that the relative motion of material is much less than in simple dried suspensions. Little comparative work on these lines has been done so far. Alternatively, special methods of fixation and drying may be used, which are likely to lead to less disturbance in the material. Freeze-drying offers a method of both fixing and drying which should leave the natural structures largely unchanged, except perhaps at the molecular level. The results so far of its application in electron microscopy are to some extent contradictory, probably due to the fact that tissues are extremely fragile after such drying. It appears that irradiation with a low voltage electron beam after freeze-drying stabilizes the preparation against the effect of the microscope beam, and the application of this technique may lead to a greater use of freeze-drying for electron microscopy. The alternative method of desiccation devised by Anderson (1951) has similar advantages and defects. The specimen is passed through a series of alcohol-carbon dioxide mixtures until it is perfused with the latter substance under high pressure. Conditions are then raised to the critical point, and the CO_2 allowed to evolve. In this way no phase boundary passes through the material, as it does even in freeze-drying, so that the disturbance should be minimal. However, the initial treatment of the specimen may be objectionable and cause a different type of artifact. The method is ingenious and deserves wider trial in comparative investigations.

The temptation with electron microscopy, as with any other new and brilliant technique, has been to use it in every possible direction without waiting for the careful evaluation of pitfalls and accumulation of critical experience that logic would have demanded. It may be hoped that this lack will now be made good in a series of careful "mopping-up" operations, making the widest use of comparative methods. At the moment it can only be said that the evidence so far available indicates that the degree of distortion on drying, even in the simplest procedure, is much less than originally feared; the fixation artifacts are equally, and perhaps more, dangerous, and careful attention needs to be paid to the special requirements of electron microscopy in this direction.

c. *Sectioning*

The additional hazards introduced in microtomy are those of cutting the section and of removing the embedding material. Knife marks are usually immediately obvious, but may be mistaken for directional effects or even striations in the tissue unless carefully checked. Displacement of material and vacuole formation is more difficult to identify; such artifacts may be produced either by the knife or during removal of the embedding substance. The latter is simply tested by observations on unextracted sections, in which most features should still be visible except the very finest. Local knife effects, such as shredding of membranes, can to some extent be checked by comparing similar regions of tissues which have met the knife at different angles. But it is impossible to be sure whether an occasional vacuole or break in a membrane is real or not, unless it is large enough to be seen by optical microscopy. One can only be guided by the frequency with which such features are observed. As more experience is gained with thin sectioning, these uncertainities will be narrowed down if not finally eliminated.

2. ARTIFACTS OF MICROSCOPY

a. *Effect of Electron Beam*

The energy density in the electron beam is enormous and the thicker the specimen the more energy is absorbed by it, with consequent rise in temperature and possibly disruption of chemical bonds in some of its component substances. Even with the minimum beam intensity needed for viewing, it is almost impossible to prevent some thick specimens, such as protozoa, from moving so much as to disrupt the supporting film. In such cases ebullition is sometimes observed, as though tightly bound water was being driven off. But with specimens of more normal thickness, such as bacteria, viruses, or thin sections, one rarely sees any change produced by the electron beam. Occasionally a dense particle, such as occur in some bacteria, will be seen to clear when the beam intensity is increased, as if some of its contents were being evaporated. But in the normal conditions of viewing it is unusual to find any artifacts of this type. Whenever a new variety of specimen is to be examined, however, it is always as well to begin with minimum beam intensity and to watch the image carefully as the intensity is increased, in order to spot any possible changes. Dense material, such as crystals of inorganic salts, can often be troublesome but frequently volatilize completely in a strong electron beam. It is also advisable to look for signs of granulation if an unduly thick layer of shadowing metal has been deposited, or when a new shadowing metal is being tried.

Although visible artifacts are rarely produced by the electron beam, it should be remembered that molecular changes are usually if not always produced. Biological material is reduced entirely to a form of graphite in an intense beam (König and Winkler, 1948), and milder irradiation probably causes cross-linkage in long chain compounds, leading to radical changes in chemical constitution as observed with high energy irradiation (Charlesby, 1953). It appears that, in consequence, most staining reactions cannot be carried out in a specimen that has been exposed to the electron beam, but at least one exception to this rule has been observed (Brieger *et al.*, 1953).

b. *Artifacts of Imaging*

As already pointed out (Section I, 3) objects that are so small as to be close to the resolution limit of the electron microscope may not be imaged in their true shape. Polygonal particles will have their corners rounded, and beyond a certain minimum size will all look circular in section. The appearance of a particle of size less than the resolution limit cannot, therefore, be any guide to its true shape. Two particles separated by less than the resolved distance will give even more misleading images, appearing as an hour-glass figure of increasing waist the closer they are together. It needs mentioning that these artifacts may appear in any micrograph, and are possibly more of a pitfall in routine work at moderate resolution then in special investigations at very high resolving power, when the operator would in any case be examining his pictures with care.

It should be hardly necessary to say that misleading appearances may also arise from experimental accidents, such as shrinkage of the support film during or before shadowing, giving rise to ridges that might be mistaken for filaments. Granulation of metal in shadowing, or in the electron beam, contamination of solvents or distilled water, and settling of airborne particles on specimens must always be looked for; one case is on record of carbon black from a shadowing filament being mistaken for chromosomes.

3. Optical Checks

In conclusion it must be emphasized that electron microscopy is in no sense a method to be relied upon to give the answer to any morphological problem in microbiology without further question. It should always be employed as one research tool among many that can be brought to bear upon such problems. The evidence of the others must also be collected and compared with that of electron microscopy. Optical observations especially should be carried out, to give an independent check on artifact formation as far as their resolution allows. Ordinary light and ultraviolet methods can be very useful, but experience has shown that phase contrast microscopy gives a better, because a visibly similar, comparison. Its contrast scheme is basically the same as that of an electron image, so that the two pictures give very similar information about a biological specimen. Indeed, at the same magnification (say, ×1500) it is often difficult to distinguish one from the other, if the phase contrast "halos" are faint. The information which this technique gives on the living organism makes it an invaluable supplement to electron microscopy.

It is also necessary to plead for open-mindedness in comparing electron micrographs with the results of the established optical methods. Long familiarity with them unconsciously instils the feeling that they show exactly "what the specimen really looks like." In the case of stained specimens obviously, but to some extent also in non-stained preparations, this cannot be true, since there is always the possibility of artifact even in optical preparations. The greater danger of artifact formation in electron microscopy should not blind one to the fact that it gives pictures that are valid within their own limitations. It is only necessary to realize that these are different limitations from those of optical methods. In short, each technique provides evidence of value, each gives a partial view of the constitution and structure of the specimen under examination, and it is the task of the research worker to correlate critically all the clues and deduce from them a coherent answer to the puzzle set by Nature.

XIII. Other Electronic Methods of Microscopy

Several techniques have been recently developed to the stage where they promise to be of value for biological investigations, and a brief indication is given here of their nature and applicability.

1. Reflection Electron Microscopy

A method of investigating the surface relief of solid specimens with a reflected electron beam, originally tried by von Borries (1940), has now been proved to be of wide applicability; for a review of recent work see Cosslett (1952). A normal electron microscope can be adapted for the purpose by arranging a wider tilt of the electron gun, up to 10° or so from the axis, and providing an object-holder with means of rotating the specimen about an axis in its surface and across the beam. When the incident beam grazes the surface at an angle of a few degrees, the electrons scattered at a similar angle can be collected and imaged in a normal train of electron lenses (Fig. 22). The image is foreshortened according to the angle that the surface makes with the lens axis. This leads to unequal magnification and resolution along and across the surface, but has the advantage of showing the relief with great clarity, as in a landscape lit by the setting sun.

The method has been used mainly for studying metal surfaces, but it has been lately shown (Menter, 1953) that many biological specimens can be examined if first given a thick coating of a highly conducting metal such as silver. The surface form of fibers has been studied successfully in this way. It is clear that a great variety of problems is open to solution, but only to a resolving power some ten times worse than that of transmission electron microscopy. The chromatic aberration arising from energy loss of the scattered electrons has so far limited resolution to about 400 Å, and it is unlikely that a value less than 100 Å will be attainable for some time, if at all. On the other hand, small differences in height on the surface can be observed, down to the order of 30 Å.

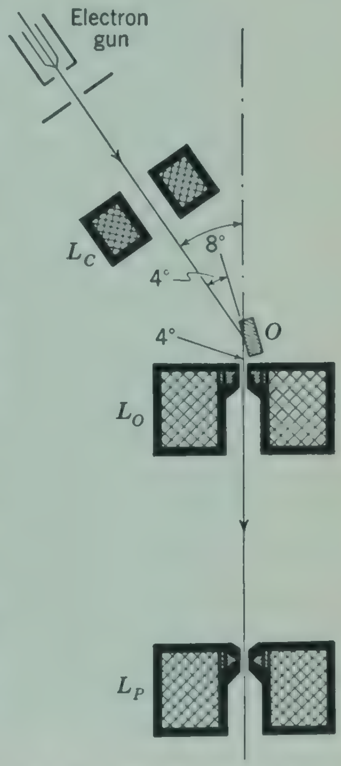


Fig. 22. Schematic diagram of reflection electron microscopy.

2. The Scanning Electron Microscope

An alternative way of investigating a surface is to direct on to it a fine point-beam of electrons, produced by demagnifying the electron source with lenses. The beam is scanned over the surface, the electrons scattered at a given angle picked up and amplified in a multiplier tube, and the output signal used to modulate a display tube in synchronism with the original scan. Such a "television" system was tried by von Ardenne (1938) and by Zworykin *et al.* (1942), but with apparatus that required about 30 min to obtain a picture. It has been recently carried out with more sensitive means, and pictures obtained on a long delay screen fast enough to give good visible results (MacMullan, 1953). The method has some advantages over that of reflection electron microscopy, especially in permitting contrast to be enhanced electronically and in giving less heating of the specimen. But the resolving power is no better and there is the great disadvantage that a special type of microscope and recording system is required.

A rather different scanning method has been devised by Möllenstedt and Düker (1953), in which a primary ion beam releases electrons from the bombarded surface. These are drawn off in a vertical direction and imaged in electrostatic lenses, so that foreshortening is avoided. Resolution also should be higher, as chromatic aberration is negligible.

3. X-Ray Microscopes

a. Reflection Type

The reflection of x-rays from curved mirrors at grazing incidence has been used to form images by several authors (Ehrenberg 1949; Kirkpatrick and Baez 1948; Lucht and Harker 1951). The advantage is obtained of having the specimen in atmospheric conditions, so that living material can be examined. Resolutions approaching 1 micron have been obtained, but it seems that the aberrations of the system are such that the limit of the ultraviolet microscope is unlikely to be exceeded even with specially figured mirrors (Dyson 1952).

b. Shadow Projection Type

A point source of electrons, as used in the electron scanning microscope, can be used to produce a microfocus of x-rays in a metal foil that is both

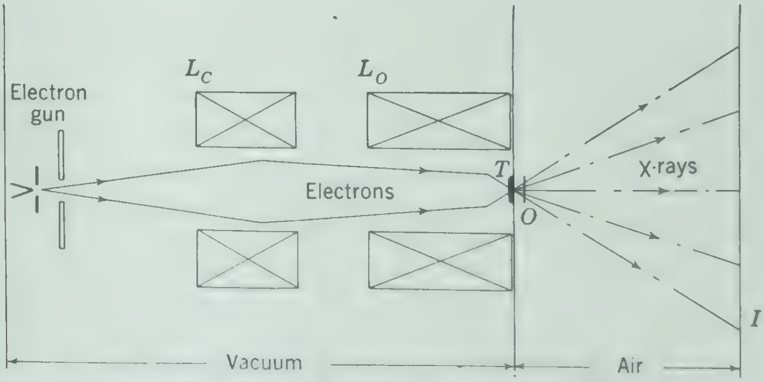


FIG. 23. Schematic diagram of x-ray shadow projection microscope.

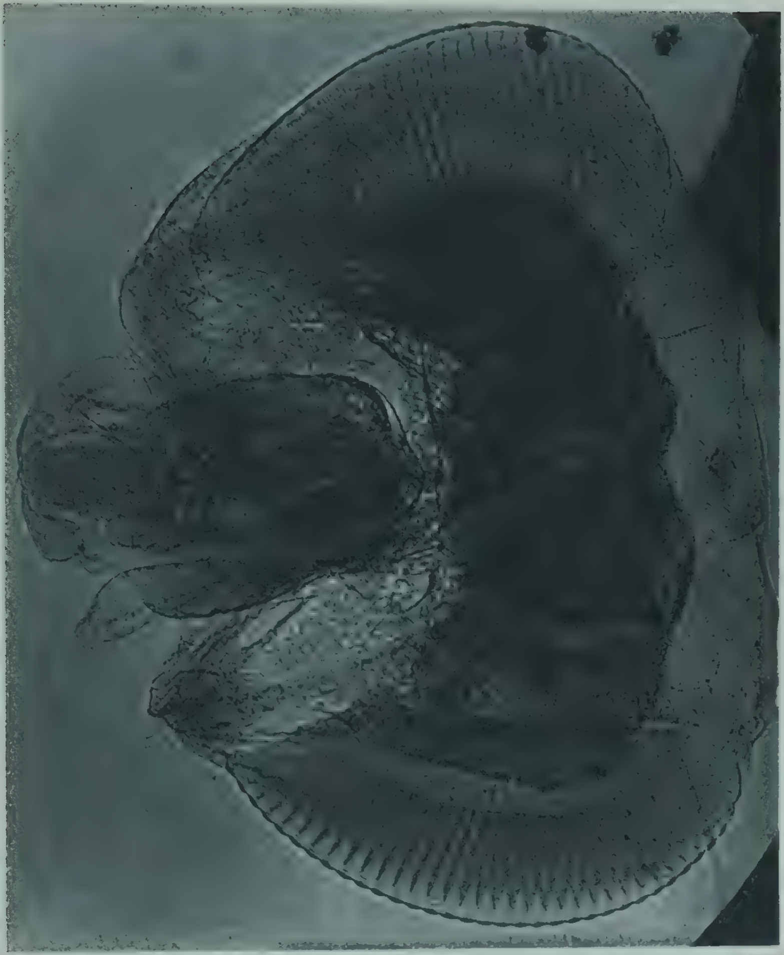


FIG. 24. X-microradiograph of head of *Drosophila melanogaster* (Cavendish Laboratory), ×200.

target and window of the tube (Fig. 23). The x-rays cast an enlarged shadow image of a specimen placed close to the window. With an object distance of 1 mm, an image distance of 5 cm gives a primary magnification of 50× and an exposure on a normal lantern emulsion of about 1 min. Further photographic enlargement of 10–20× allows a final magnification of 500–1000×. The method again allows biological specimens to be examined in atmospheric conditions, although best resolution is obtained with desiccated specimens. With 10kv electrons the best results from insects show a resolution better than 0.5 micron (Fig. 24), thus already approaching that of the optical microscope (Cosslett and Nixon, 1952, 1953). It is probable that the use of the method with living specimens is limited by the high dosage needed to obtain photographs, but in itself the study of the lethal effects of x-rays on organisms is of great interest. The method is potentially capable of a resolving power exceeding that of the ultraviolet microscope, but at the cost of longer exposure times. In the 1 micron range, on the other hand, it gives much shorter exposures than those obtaining in direct microradiography with fine grained emulsions (Engstrom, 1950). The latter method in any case cannot give a better resolution than that of the optical microscope which must be used to enlarge the micro-image obtained.

c. *Scanning Type*

The scanning electron microscope can be adapted to x-ray working by allowing the "flying-spot" to scan across a window-target, outside which is placed the specimen. The x-rays transmitted through each picture point are registered in a counter tube, the output of which is amplified and applied to a display tube. Such a system has been proposed by Pattee (1953) and is now being tested. Its success will depend in large part on the availability of a sufficiently powerful electron beam. The main advantage over the shadow projection method is that the contrast is under electronic control and that the target heating is less, just as in the scanning electron microscope.

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